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**TUBERCULOSIS IN CHILDREN:
DIAGNOSIS AND EPIDEMIOLOGY**

by

TRAN THI SINH

A thesis submitted to the Open University U.K

For the degree of Doctor of Philosophy in the field of Life Sciences

Oxford University Clinical Research Unit

Ha noi, Viet Nam

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Abstract

Globally, an estimated one-million new tuberculosis (TB) cases occurred in children in 2014. For a long time, research of TB in children has been neglected. Most research and surveillance of TB is performed in adults, and the resulting lack of evidence in children is a major barrier for implementation of rational control strategies for children, including diagnosis. More research on TB in children is of importance as children are more susceptible to developing severe extrapulmonary TB, children require different approaches to both diagnosis and treatment and paediatric TB reflects the ongoing transmission of TB in the population. This research gap on the epidemiology of tuberculosis in children, especially in high burden countries, should be addressed in order to better understand the dynamics of TB transmission in both adults and children. Accurate data are the basis for establishment of effective control strategies. This thesis aims to assess the diagnostic role of microscopic observation drug susceptibility (MODS) and mycobacterial blood culture for diagnosis of TB in children as well as to present the epidemiological characteristics of paediatric TB in northern Vietnam with regard to drug resistance and genotypes of *Mycobacterium tuberculosis* (MTB), the causative agent of TB, isolated from them.

MODS is a low cost non-commercial liquid culture assay to detect MTB by microscopy. MODS was compared with conventional assays including Ziehl-Neelsen smear (ZN) and Löwenstein-Jensen culture (LJ) in a study conducted from 2009 to 2010 at the National Hospital for Paediatrics, a general paediatric hospital, in Hanoi, Vietnam. In suspected paediatric TB cases, the MODS test was shown to be significantly more sensitive than both smear (46.0% vs. 8.8%) and LJ (46.0% vs. 38.9%), and significantly faster than LJ with a median time difference of 28 days in favour of MODS (7 days vs. 35 days). The findings

suggest that MODS is a rapid low-cost diagnostic tool for TB diagnosis in the paediatric population.

The additional yield of mycobacterial blood culture was assessed in comparison to routine detection methods for TB diagnosis in children in two hospitals in Vietnam. The findings show that mycobacterial blood culture detected an additional six TB cases of which 5 cases were negative with other tests and in the remaining case no other tests were done. All six cases were susceptible to rifampicin and isoniazid. The overall performance of TB blood culture was poor as compared to routine culture with regard to detection rate (2.9%, 16/554 vs. 16.6%, 92/554) and turnaround time (26 days vs. 14 days). The incremental cost for adding one additional TB case is substantial. Therefore, addition of mycobacterial blood culture into routine diagnostics has limited utility in resource limited settings.

To assess the molecular epidemiology of paediatric TB in northern Vietnam, a collection of 125 MTB isolates from children with TB admitted to NHP during 2009 to 2013 was analysed. Drug susceptibility testing results from 121 isolates and genotypes from 120 isolates were generated. The phenotypic drug susceptibility testing showed that 20.7% was resistant to isoniazid (25/121), 3.3% resistant to rifampicin (4/121), 28.1% resistant to streptomycin (34/121) and 2.5% resistant to ethambutol (1/121). There were 4 cases with multidrug resistant TB. The high frequency of resistance to isoniazid and streptomycin are consistent with data from adults in Vietnam, suggesting the ongoing transmission of drug resistant MTB in the community. MIRU typing patterns showed that the Beijing genotype was predominant in this population (63.3%, 76/120), which is in agreement with various prior studies in adults in Vietnam. These findings provide more evidence to support the hypothesis of the epidemic spread of the Beijing genotype in Vietnam. In this study, an association between Beijing genotype and drug resistance to streptomycin and isoniazid was observed. The number of

MDR isolates was too small to draw conclusions regarding association of MDR and Beijing genotype.

Collectively, these results demonstrate that liquid culture can improve the yield of TB diagnosis in Vietnam and mycobacterial blood culture should not be routinely performed for paediatric cases. The molecular epidemiology study also showed that the Beijing genotype is the predominant lineage among actively transmitted strains in Vietnam and that it is associated with both isoniazid and streptomycin resistance. Paediatric TB remains a significant cause of morbidity and mortality in Vietnamese children and sustained political and social commitment from all stakeholders, including governments, funders, academics and the medical community will be needed to improve diagnosis, treatment and prevention of TB in children globally.

Co-Authorship

Tran Thi Sinh, the author of this work, was responsible for conducting the experiments, supervising patient enrolment, data analysis and writing of all the studies in this thesis, with exceptions of the work detailed below

The molecular testing for drug resistance by Multiplex allele-specific PCR was performed by Ms Tran Dinh Dinh, at the Oxford University Clinical Research Unit in Ho Chi Minh City, Vietnam.

The phenotypic susceptibility testing of MTB isolates was performed by Pham Thi Thu Huyen at the Microbiology Department and National TB Referral Laboratory of the National Lung Hospital, in Hanoi, Vietnam.

Publication

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Abbreviations

AFB	Acid fast bacilli
BCG	Bacillus Calmette-Guérin
CSF	Cerebrospinal Fluid
DR	Drug resistance
EPTB	Extrapulmonary tuberculosis
ETB	Ethambutol
HIV	Human Immunodeficiency Virus
INH	Isoniazid
LJ	Löwenstein–Jensen
MAS-PCR	Multiplex allele-specific PCR
MDR TB	Multidrug resistant tuberculosis
MGIT	Mycobacterial Growth Indicator Tube
MIRU	Mycobacterial Interspersed Repetitive Units
MODS	Microscopic Observation Drug Susceptibility
MTB	Mycobacterium tuberculosis
NHP	National Hospital of Paediatrics
NLH	National Lung Hospital
NTM	Non tuberculous meningitis
OADC	Oleic acid Albumin Dextrose Catalase
OUCRU	Oxford University Clinical Research Unit
OxTREC	Oxford Tropical Research Ethics Committee
PANTA	Polymyxin Amphotericin B Nalidixic acid Trimethoprim Azlocillin
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PTB	Pulmonary tuberculosis
RIF	Rifampicin
Spoligotyping	Spacer oligonucleotide typing
SM	Streptomycin
TB	Tuberculosis
TBM	Tuberculous meningitis
TST	Tuberculin skin test

VNTR	Variable Number of Tandem Repeats
WHO	World Health Organization
ZN	Ziehl–Neelsen
AFB	Acid fast bacilli
BCG	Bacillus Calmette-Guérin

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Chapter 1

Introduction

1.1 History of tuberculosis

Humans have suffered from tuberculosis (TB) since ancient times and TB has been described in ancient texts from China about 2,300 years ago (2). Fragments of the genome of *Mycobacterium tuberculosis* (MTB) - the causative agent of tuberculosis have been detected in a 3,300-year-old Egyptian mummy (3). The tuberculosis epidemic reached a peak in Europe and North America in the 18th and 19th century, and scientists started to focus more on tuberculosis pathogenesis (2). In 1865, the French doctor Jean Antoine Villemin showed that tuberculosis could be transmitted from a tuberculous animal to a healthy animal in an inoculation experiment. Robert Koch discovered that MTB is the actual infectious agent of the disease. He also developed the first staining method to detect MTB, exploiting the acid-fast property of the bacilli. His announcement of this seminal discovery on 24th March 1882 is still celebrated as World TB day (4). In 1907, the tuberculin skin test was developed and the Bacillus Calmette–Guérin (BCG) vaccine was created by serial passage of *Mycobacterium bovis* in the laboratory to create an attenuated vaccine strain, which has been widely used since 1921. Vietnam, when colonised by the French, was one of the first countries where the vaccine was tested by French researchers. Unfortunately, efficacy in reported studies varied widely (0-80%) for unknown reasons, which may include variation in ethnicity, prior exposure to other mycobacteria in study populations, or variation in the vaccine strains themselves (5, 6). Although the protective effect of BCG vaccine is limited, it is widely used for perinatal vaccination in endemic countries today because it prevents the most severe forms of TB in children, especially tuberculous meningitis and miliary TB (5-8).

Along with understanding the disease, the search for a cure became a priority. Before the discovery of the first anti-TB drug in the 1940s (9), the only available options for TB treatment were sanatoria and surgery. The experience of European sanatoria was described in Thomas Mann's novel "Der Zauberberg" (The Magic Mountain), for which he won the Nobel Prize of Literature in 1929 (10). There were many 'false-dawns' in the search for an effective TB treatment, with the most famous being sanocrysin and gold therapy (11, 12). The treatment trials of streptomycin are widely acknowledged to be the first randomised trials of a drug. The joy at initial results of streptomycin treatment was quickly tempered by the realisation that the relapse rates after mono-therapy were high, due to the emergence of streptomycin resistance (13). In the following decades, several new antibiotics were discovered: Para-aminosalicylic acid (PAS in 1944), Isonicotinic acid hydrazide (Isoniazid, INH in 1952), Pyrazinamide (PZA in 1954), Cycloserine (in 1955), Ethambutol (EMB in 1962) and Rifampicin (RIF in 1963).

A landmark series of trials by the Medical Research Council (UK) and the International Union against TB and Lung Disease (IUATLD, The Union) gradually developed and refined the treatment of TB using combination therapy (13). The addition of rifampicin and pyrazinamide eventually led to the establishment of a 6-month regimen, termed 'short-course'. An 'intensive phase' of treatment is used for the first 2 months with four drugs: isoniazid, rifampicin, ethambutol and pyrazinamide. This is followed by a 'continuation phase' of isoniazid and rifampicin in the final four months (14). Variations of this regimen were used with some countries adopting an 8-month regimen and thioacetone widely used in Africa due to low cost (15). The eight month regimen was shown to be inferior to the 6-month regimen in a trial conducted by the The Union, and is no longer recommended by WHO (16, 17). Thioacetone causes a high rate of severe adverse events in people living with HIV and has therefore been discontinued for TB therapy (15). Interest in TB drug discovery evaporated

after the 1960's and no novel anti-TB drugs were discovered in subsequent decades until the development of the second and third generation fluoroquinolones in the 1980's (18). However, resistance began to emerge and spread worldwide and by the millennium it was recognised that novel anti TB drugs were an urgent public-health priority, to prevent a return to 'untreatable' TB (19).

A major research drive has resulted in the approval by the US food and drug administration (FDA) of two new drugs for MDR TB treatment (bedaquiline and delamanid). Importantly, the mechanism of action of these drugs differs from that of existing TB drugs (20). Despite progress made during the last decades, the pipeline of anti-TB drug development is lagging. The need for new combination therapies to treat drug resistant forms and the need for shorter treatment regimens for all forms of TB remains urgent.

The last decade has seen major advances but also major disappointments in TB research. The Foundation for Innovative New Diagnostics (FIND) has led the systematic evaluation of TB diagnostic methods, which, combined with research by other groups, has led to large number of evidence-based recommendations from WHO on diagnostic strategies for TB, after long stagnation. The use of nucleic acid amplification tests (NAAT) for TB diagnosis has greatly increased following the endorsement by WHO in 2010 of the GeneXpert MTB/RIF test (21-23). This test allows the rapid detection of both MTB and resistance to rifampicin, but has a number of important limitations, which stop it being a true point-of-care test (24). Three trials of shortened (4-month) regimens including a fluoroquinolone all failed to show efficacy in 2013, and the first trial of a new TB vaccine also failed in that year (25-28). However, the Millennium Development Goals (MDG) for TB control stimulated a renewed focus and intensification of control efforts using existing tools. Global TB mortality has fallen by 47%

since 1990, with almost all of that gain post-millennium, and most countries are now seeing a decline in incidence of 1-2% per year (1).

Paediatric TB has long been neglected as a research area. It was long assumed that control of adult TB would naturally eliminate paediatric TB, and the lack of systematic data collection on paediatric TB has obscured the problem. The last decade has seen a series of calls for intensified research on paediatric TB and a push by WHO for systematic age disaggregated data collection (1, 29, 30). Attempts have been made to develop standardised research case-definitions to improve assessment of new diagnostics (31, 32), paediatric TB is now included in systematic reviews of new TB interventions, children have been included in a number of recent and planned clinical trials (33) and new much-needed paediatric TB drug formulations were developed in 2015 (31, 32, 34).

In 2015 the new END TB strategy of WHO set targets for TB elimination for the first time (Table 1.1). To achieve this ambitious goal, we will need new drugs, diagnostics and vaccines.

Table 1.1 The End TB strategy at a glance (2016-2035) (35)

Vision	A world free of TB – zero deaths, disease and suffering due to TB			
Goals	End the TB pandemic			
Indicators	Milestones		Targets	
	2020	2025	SDG 2030 ^a	2035
Reduction in number of TB deaths compared with 2015	35%	75%	90%	95%
Reduction in TB incidence rate compared with 2015	20% ($<85/10^5$)	50% ($<55/10^5$)	80% ($<20/10^5$)	90% ($<10/10^5$)
TB-affected families facing catastrophic costs due to TB	0%	0%	0%	0%
Principles				
<ol style="list-style-type: none"> 1. Government stewardship and accountability, with monitoring and evaluation. 2. Strong coalition with civil society organizations and communities. 3. Protection and promotion of human rights, ethics and equity. 4. Adaptation of the strategy and targets at country level, with global collaboration. 				
Pillars and components				
<i>Integrated, patient-centred care and prevention</i>				
<ol style="list-style-type: none"> A. Early diagnosis of TB including universal drug-susceptibility testing and systematic screening of contacts and high-risk groups. B. Treatment of all people with TB including drug-resistant TB and patient support C. Collaborative TB/HIV activities and management of co-morbidities. D. Preventive treatment of persons at high risk and vaccination against TB 				
<i>Bold policies and supportive systems</i>				
<ol style="list-style-type: none"> A. Political commitment with adequate resources for TB care and prevention. B. Engagement of communities, civil society organisations and public and private care providers. C. Universal health coverage policy and regulatory frameworks for case notification, vital registration, quality and rational use of medicines and infection control. D. Social protection, poverty alleviation and actions other determinants of TB 				
<i>Intensified research and innovation</i>				
<ol style="list-style-type: none"> A. Discovery, development and rapid uptake of new tools, interventions and strategies. B. Research to optimise implementation and impact and promote innovations 				

a: Targets linked to the Sustainable Development Goals (SDGs)

1.2 Biology of *Mycobacterium tuberculosis*

MTB, the causative agent of tuberculosis, belongs to the *Mycobacterium tuberculosis* complex (MTBC) within the family of Mycobacteriaceae (36, 37). The MTBC consists of *Mycobacterium africanum*, *Mycobacterium bovis*, *Mycobacterium canettii*, *Mycobacterium microti*, *Mycobacterium tuberculosis*, and others.

Mycobacteria are aerobic, non-mobile and non-sporulating bacilli with a relatively slow replication rate compared to other bacteria (about 24 hours per generation). It possesses a unique cell wall structure (38), which is rich in unusual lipids, glycolipids and polysaccharides (39). The mycolic acid, the major lipid compound in the cell wall, is strongly hydrophobic, allowing the bacteria to survive in the macrophage and resist hydrophilic drugs and dehydration (40, 41). The high lipid content of the cell wall renders MTB resistant to decolourisation by acid after being stained with aniline dyes like carbol fuchsin, which is the basis of the ZN staining.

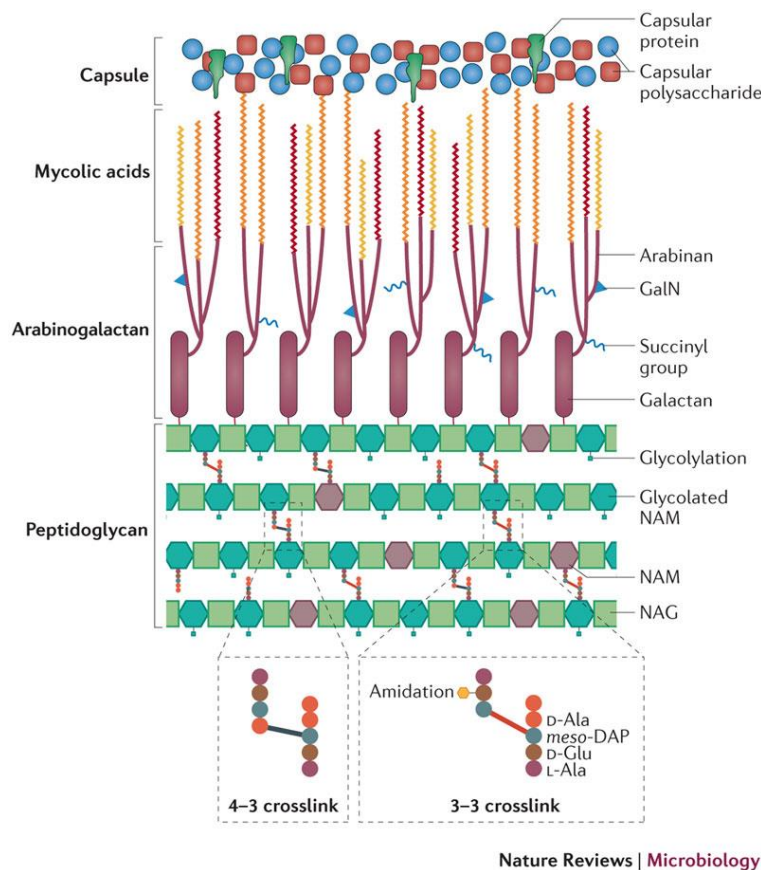


Figure 1.1 The cell envelope of mycobacteria (42).

Mycolic acids are the major lipids found in the cell envelope of species in the mycobacteria species.

1.3 Epidemiology of global tuberculosis

Tuberculosis remains a major global health problem. MTB infects approximately one third of the world population. MTB is among the leading cause of death from infectious diseases globally. In 2014, an estimated 9.6 million people developed disease and 1.5 deaths from TB were recorded (43). TB is present in all regions of the world with incidences and death rates varying per country (Table 1.2) (Figure 1.2). Approximately 80% of all TB cases occur in 22 high burden countries with more than half (58 %) in South-East Asia and the Western Pacific Region. Globally, the overall TB incidence has been decreasing at an average rate of about 1.5% per year between 2000 and 2013. However, there is a gap of one third between the

number of notified TB cases and the estimated number of TB cases, which needs a major effort to close. In 2014, an estimated 3.6 million cases were either not diagnosed, or diagnosed but not reported to national TB control programs (43). Tuberculosis is also a major problem in risk groups like HIV infected individuals. HIV positive individuals are more susceptible to TB infection and have an increased risk to develop active TB following infection (approximate 30 times higher than HIV uninfected people) and are more likely to fail treatment. In 2014, WHO reported an estimated 12% of 9.6 million TB cases living with HIV and an estimated 400,000 deaths from TB among HIV infected people (1, 43).

Another challenge to the global TB control effort is the increasing number of cases resistant to the drugs used for treatment. Multidrug resistant tuberculosis (MDR-TB) is caused by bacteria that are resistant to at least the two most powerful anti-TB drugs: isoniazid and rifampicin. Extensively Drug-Resistant-TB (XDR-TB) is a form of TB caused by MDR-TB bacteria additionally resistant to a fluoroquinolone and at least one of three injectable drugs (amikacin, kanamycin, or capreomycin). Totally drug resistant TB (resistant to all known TB drugs) has been described in the literature but the term is not accepted by WHO due to the difficulty of standardising *in vitro* susceptibility testing for second line drugs (44). Available WHO global data of drug resistance show that an estimated 3.5% of new and 20.5% of previously treated TB cases had MDR-TB in 2013. Eighty-five percent of these MDR cases occurred in 27 countries, with the highest incidences found in a number of former Soviet-Union states. Globally, an estimated 9% of MDR-TB cases were XDR-TB. Drug resistance is a significant cause of treatment failure. Worldwide, the treatment success for MDR-TB cases notified and enrolled to treatment was just 48% in 2011, around half of the 86% success for new mostly susceptible TB cases in 2013 (43). The majority of MDR-TB is not detected or treated with appropriate regimens, despite large improvements in MDR-TB case detection following the introduction of GeneXpert MTB/RIF testing.

Estimated TB incidence rates, 2013

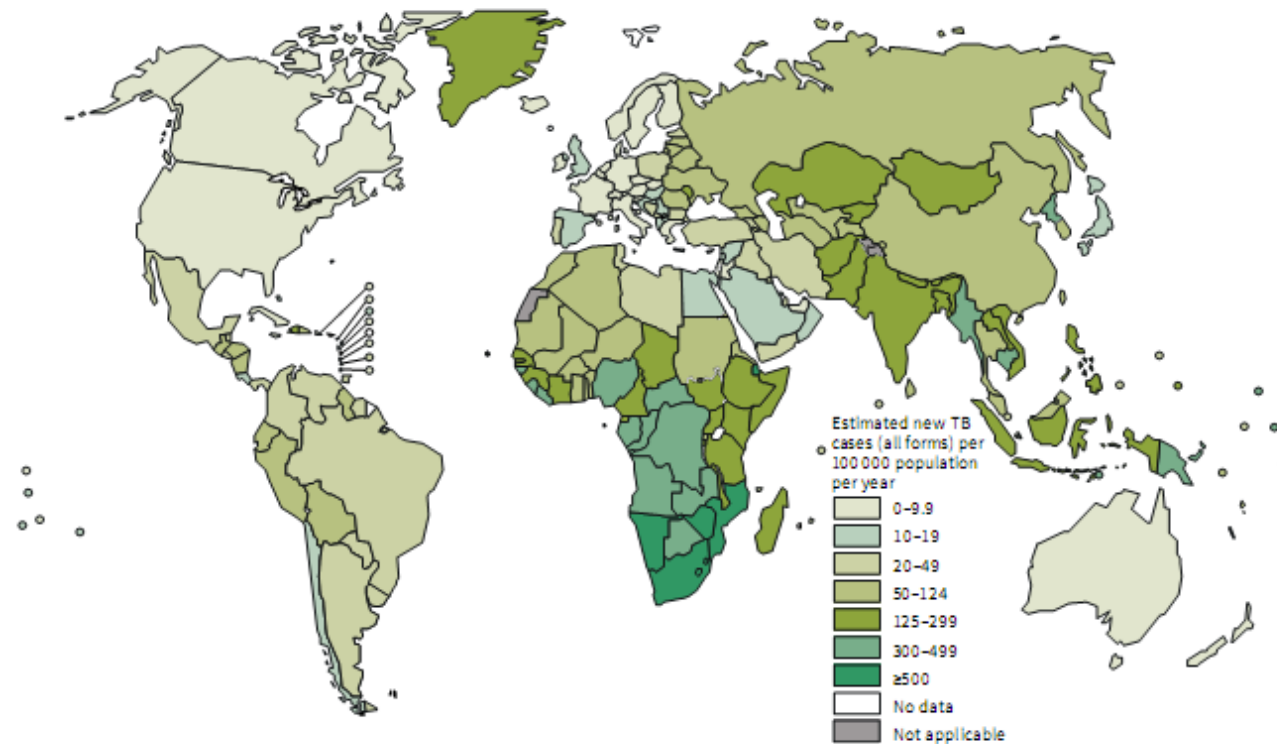


Figure 1.2 Estimated TB incidence rates by countries in 2014 (43)

Table 1.2 Estimated WHO Regional TB statistics for 2014

Reproduced from Global TB report 2015 (43)

Region	TB deaths (HIV -ve only)	HIV/TB deaths	Total incident TB cases	New cases
Africa	450,000	310,000	3,200,000	2,700,000
America	17,000	6,000	350,000	280,000
Easter Mediterranean	88,000	3200	1,000,000	740,000
Europe	33,000	3200	440,000	340,000
South-East Asia	460,000	62,000	5,400,000	4,000,000
Western Pacific	88,000	4900	2,100,000	1,600,000
Global Total	1,100,000	390,000	13,000,000	9,600,000

* The deaths figures exclude the deaths of people who had both TB and HIV infection at the time of their death

Percentage of new TB cases with MDR-TB^a

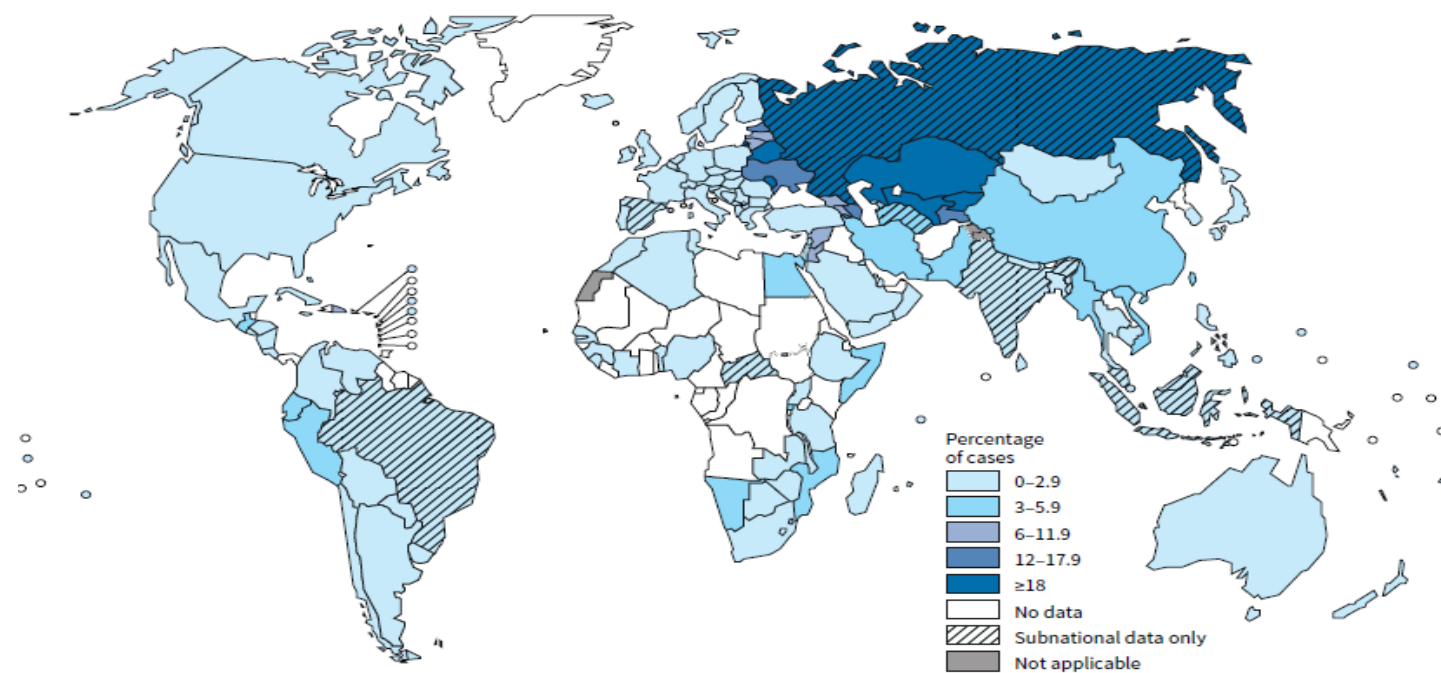


Figure 1.3 Percentage of new TB cases with MDR-TB in 2013 (1, 43)

1.4 Pathogenesis of tuberculosis (45)

When people come into contact with an infectious (smear-positive) TB case, they may inhale droplets / aerosols containing MTB. These droplets are formed when an active TB patient coughs or sneezes. The droplet travels into the lung and may reach the alveoli, where the bacteria are recognised and ingested by macrophages (46). Subsequently, the localised inflammatory response occurs, recruiting mononuclear and other cells of the immune system from the surrounding blood vessels. There are three possible outcomes to MTB within the macrophage: (1) MTB infection may progress to primary TB disease, (2) be eliminated or (3) become dormant (latent TB, LTBI). LTBI may reactivate after several years or decades to cause disease, usually when the infected individual becomes immune compromised due to age, malnutrition, illness or immunosuppressive therapy (47).

In most cases (90%), the immune response contains the MTB bacilli in a dormant state, in which active replication and tissue damage are prevented and no clinical symptoms or signs of active TB disease are observed. The infection is controlled and rarely progresses to disease, so called latent infection. In a small number of cases, if the immune system failed to control, the bacilli will multiply and cause active TB and may spread into the surrounding area and distant organs via the blood stream or lymphatic system. The progress from latent infection to TB disease can occur anytime in person's life depending on age and immune system status (48). As pulmonary disease progresses, granulomas, the hallmark of TB disease, begin to form in the lungs as a result of the immune response to the invasion (46, 49). If TB remains untreated, cavities will form and lung function is significantly impaired, eventually resulting in death in the majority of cases. The factors responsible for successful containment of infection or progression to active disease are not completely understood. Significant co-

morbidities such as HIV and diabetes and the use of immunosuppressive or anti-inflammatory drugs are well-documented risk factors for progression to active disease, but in apparently healthy individuals the risk factors leading to disease are less well understood. Smoking, chronic obstructive pulmonary disease (COPD) and other lung impairment is a risk factor (50, 51).

1.5 Paediatric tuberculosis

1.5.1 Overview of paediatric tuberculosis

Globally, WHO estimated that 1 million cases of TB occur among children (approximate 10% of the total 9.6 million TB cases) with 140,000 deaths based on an extrapolation from estimates of adult TB (43). Approximately 75% of global childhood TB cases occur in the 22 high TB burden countries (52), where childhood TB incidence contributes up to 20% of total TB cases (53). Dodd *et al* used a mathematical modelling technique, to estimate that 650,977 children developed TB in 2010 (52). Although childhood TB contributes to the morbidity and mortality of children (54-56) and represents a large TB reservoir, it has not been a priority for national TB control programmes. One of the reasons for this is that TB in children is difficult to diagnose (and therefore both under-diagnosed and unconfirmed in many cases) and is considered to be less infectious than adult TB (23, 57). Recently, childhood TB is gaining more attention as it contributes considerably to childhood morbidity and mortality and there have been several research agendas and calls to action for childhood TB (58-63).

Following exposure to an infectious TB case, children, like adults may successfully eradicate the infection or may become infected with MTB and subsequently may contain the bacilli but not eradicate them (latent TB) or develop symptomatic active TB. The risk to develop active disease varies by age (64) and is highest in very young children and children with immunosuppression, such as HIV infection (22) or malnutrition (65-67) (Table 1.3). Disease

progression after infection occurs within 12 months in the majority of children (>95%) who develop disease. Therefore, most childhood TB reflects ongoing transmission, and the pattern of drug resistance observed in children provides an accurate estimate of primary drug resistant TB within communities. Children can therefore be considered a sentinel population, with childhood TB incidence as an indicator to measure the effectiveness of TB control programmes (68).

Like adult TB, TB can occur in any site of the body in children, but mainly occurs in the lungs (pulmonary TB) (69). Following inhalation to the lungs, MTB bacilli may disseminate to other organs via haematogenous spread, causing extra-pulmonary TB (EPTB) (Figure 1.4). Cervical lymphadenitis (also known as scrofula or The King's Evil) is the most common phenotype of extrapulmonary TB for children, followed by tuberculous meningitis. Miliary TB and tuberculous meningitis are among the most severe EPTB presentations. Other extrapulmonary sites are described less commonly including the eyes, larynx, middle ear, mastoid, genitourinary and intestinal tract, pleura, skin etc. Factors contributing to disease presentation include: the age at time of primary infection and the time elapsed since infection. Very young children and immunosuppressed children are more at risk of severe TB. Children may also acquire infection with *Mycobacterium bovis* through the drinking of unpasteurised milk and "BCG-osis" may occur in children with HIV, following BCG vaccination. For this reason BCG vaccination is not recommended in known HIV positive children (70).

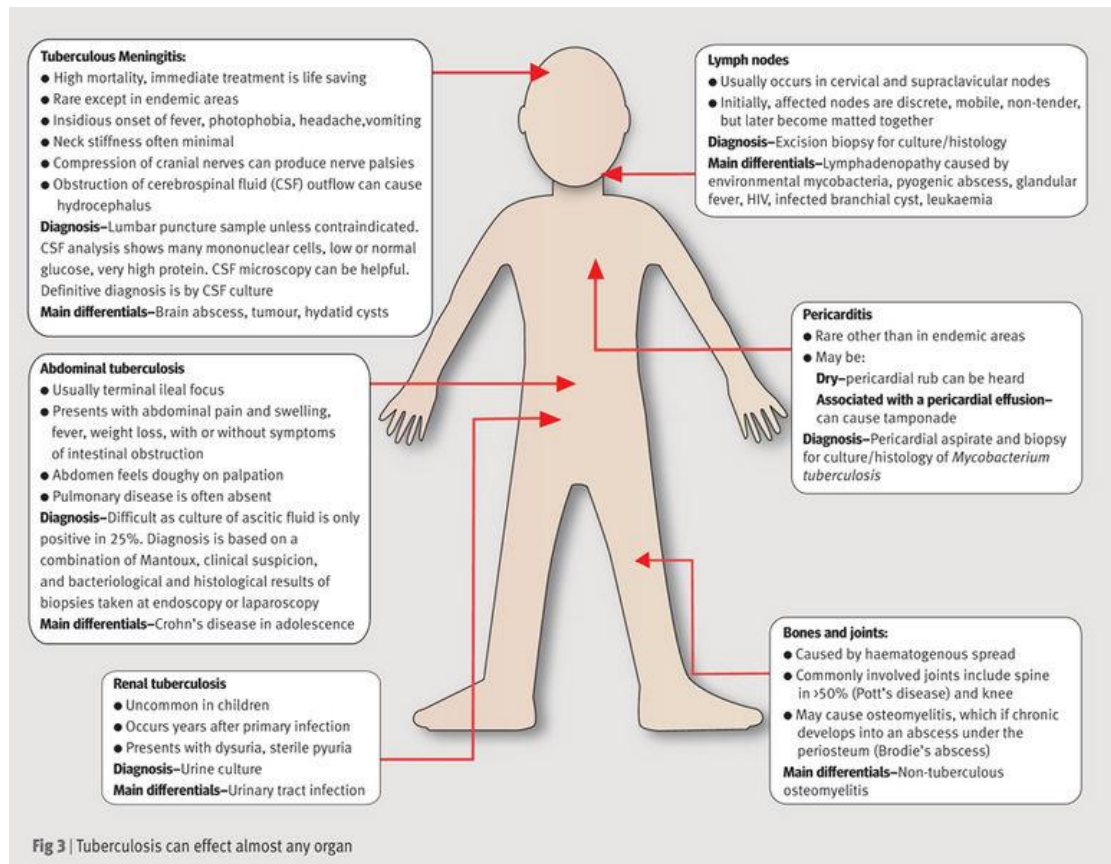


Figure 1.4: Disease manifestations of tuberculosis in a child

Reproduced from:

https://microbewiki.kenyon.edu/index.php/Tuberculosis_in_Children_in_Developing_Countries

Table 1.3 Risk of pulmonary and extra-pulmonary TB in children following infection with MTB

Reproduced from Ben Marais 2004 (65).

Age at primary infection	Immunocompetent children	Risk of disease following primary infection (%)
< 1 year	No disease	50
	Pulmonary disease	30-40
	TBM or miliary disease	10-20
1-2 years	No disease	70-80
	Pulmonary disease	10
	TBM or miliary disease	2-5
2-5 years	No disease	95
	Pulmonary disease	5
	TBM or miliary disease	0.5
5-10 years	No disease	98
	Pulmonary disease	2
	TBM or miliary disease	<0.5
> 10 years	No disease	80-90
	Pulmonary disease	10-20
	TBM or miliary disease	<0.5

1.5.2 Diagnosis of tuberculosis in children

The accurate and prompt diagnosis of TB in children is essential, as delayed or incorrect diagnosis may lead to disseminated disease, death or severe sequelae in children. The diagnosis of TB in endemic countries is based on physical examination, history and contact investigation and available diagnostic tools (71). Smear microscopy is usually available with chest X-ray at larger centres; MTB culture is rarely available in endemic countries outside national reference laboratories. Unfortunately, the diagnosis of TB in children is more challenging compared to adults for several reasons.

Firstly, children often have an atypical clinical presentation (including extra-pulmonary disease) and the classical triad of adult TB: cough, night sweats and weight loss is often absent. Constitutional symptoms may include failure to thrive and reduced playfulness; low-grade or intermittent fever is less frequently seen. For children with suspicion of pulmonary TB, the usual presenting symptoms are a persistent, non-remitting cough or wheeze that is unresponsive to the treatment for an alternative cause (e.g. bacterial pneumonia). However, it is difficult to distinguish between other common respiratory infections such as pneumonia. In children with HIV infection, the clinical evaluation for TB is even more challenging because it is difficult to differentiate the TB-related symptoms from those caused by other HIV associated conditions and opportunistic infections (72, 73). For example, failure to thrive or weight loss is a typical feature of both TB and HIV infection.

Secondly, collection of good quality specimens for diagnosing childhood TB is challenging. For PTB suspects, expectorated sputum is not always available in young children because they may not produce sputum or swallow their sputum. A gastric aspirate is therefore recommended for those who are unable to expectorate sputum, but the gastric aspiration procedure requires overnight-fasting, hospital admission and is invasive.

With current diagnostics, even with optimal conditions, the TB confirmation rate is extremely low, due to the difficulty to collect the appropriate specimen and the low bacterial load (paucibacillary) in paediatric specimens. Therefore, childhood TB treatment is often empiric without laboratory evidence, resulting in both over and under-diagnosis. Over-diagnosis leads to unnecessary treatment for TB, which may cause harm to children from severe adverse drug reactions, while under-diagnosis results in disease progression and increased mortality and morbidity. Therefore, improving the accuracy of diagnostics is necessary to reduce this risk.

1.5.3 TB case definition

For research on paediatric TB diagnostics, one reason for the lack of data has been the lack of a standardised research case definition. Like for adult TB, there is no gold standard (100% sensitivity and 100% specificity) against which to assess new techniques. For adult pulmonary TB, new diagnostic tools are usually assessed against culture, although it is recognised that this is an imperfect gold standard at best. In assessments of diagnostics for extrapulmonary TB and children, however, culture is known to be negative in over half of clinically treated cases. This led to each new published evaluation of TB diagnostics in children using, usually minor, variations of similar case definitions. This made it impossible to make comparative evaluations between studies and also to conduct meta-analysis of published data on each test. It is common to use a diagnostic certainty classification with microbiologically confirmed TB as a ‘definite’ diagnosis. Recently, patients with nucleic acid amplification positive results have been added to this confirmed category. The remaining cases are often classified as ‘probable’, ‘possible’ and ‘not TB’ using different criteria for the classification for whom there is inconsistent follow-up data.

In 2012 a consensus case definition for classification of intrathoracic tuberculosis in children was first published (32, 74) and this was updated in 2015 following feedback from

stakeholders (75). A standardised case definition for research on tuberculous meningitis also incorporates childhood TBM (76), but there remains a need for consensus guidelines to standardise the reporting of other forms of extra-pulmonary TB in children.

1.5.4 Specimen collection

Alternative less invasive respiratory specimens have been studied to facilitate specimen collection and diagnostics in children, including induced sputum and string test. Induced sputum (IS) is collected using hypertonic saline to irritate the airways to induce cough and obtain material from lung airways (77). Sputum induction is unpleasant for patients, but was reported to be safe and useful for MTB confirmation of PTB in both HIV-infected and HIV-uninfected children (78-80). However, the culture detection rate of IS was found to be less than of gastric aspirates (GA; 17.9% versus 32.5%) for diagnosing TB among children (81).

The string test (ST), which is a method to retrieve enteric pathogens, is a potential tool for detection of MTB in patients who are unable to produce sputum (82). The string test uses an absorbent nylon string coiled inside a gelatin capsule. One end of the string protrudes from a small hole in the capsule. The trailing part of the string is tapped to the patient's cheek and the capsule is swallowed. The capsule then dissolves in the stomach, allowing the string to become coated with gastro-intestinal secretions. After the allocated time, the string is retrieved by gentle pulling and placed in 0.9% saline solution and processed according to standard procedures for TB testing. The string test was reported to have an overall yield for culture confirmation in adults similar to IS (83). The culture yield of ST for diagnosis of TB in children was also reported to be comparable to IS in the study Nansumba *et al* of evaluating different specimen types for diagnosis of TB in children (84). However, the major disadvantage of ST is that it is not always well tolerated by young children (<4 years of age) (85).

Recently, some attention has been focused on investigating other types of specimens which are easily collected such as stool, blood or urine (86, 87).

Detecting MTB by GeneXpert MTB/RIF on stool samples from children having PTB was 47.1% sensitive as compared to the sputum MGIT culture standard (87). In a recent small study using a modified stool processing protocol, the sensitivity of GeneXpert MTB/RIF on stool was 85% of positive cases by GeneXpert MTB/RIF on IS or gastric aspirate (88). For using blood sample, in a recent study by Pohl et al (89), detection yield of MTB in the whole blood by GeneXpert MTB/RIF was reported to be limited (7.1% of sensitivity using both LJ and MGIT culture as the reference standard). The new GeneXpert MTB/RIF Ultra assay is reported to have an improved limit of detection, but is unlikely to substantially improve the sensitivity on blood sample because the sample is always paucibacillary, even in adults and inhibitors present in blood, including haemoglobin, impair the sensitivity of PCR(90) .

Collection of multiple samples has been frequently reported to increase the overall yield of detection of paediatric TB cases compared to single specimens (91). The detection rate of culture by two consecutive gastric aspirates was 67% versus 38% by single sample in diagnosing PTB among children in Cape Town, South Africa (92). Combination of different specimen types also was found to be a potential approach to improve the diagnosis TB in children (93). For instance, the sensitivity of GeneXpert/MTB RIF increased from 72.4% (only using standard samples) to 79.3% (additional samples including nasopharyngeal aspirate and stool) in an evaluation of different samples for TB diagnosis in children in multiple settings (86).

1.5.5 TB diagnostic methods

During the past decades, systematic multi-country research programs on improving TB diagnostic tools were established, primarily led by the Foundation for Innovative New Diagnostics (FIND) (Figure 1.5). The Strategic and Technical advisory Group (STAG) of WHO also began to conduct systematic review of all published studies on each technique which has led to several WHO recommendations on TB diagnosis (Table 1.4). However, the most evidence comes from adult studies and there remain questions about their usefulness in diagnosis of TB in children.

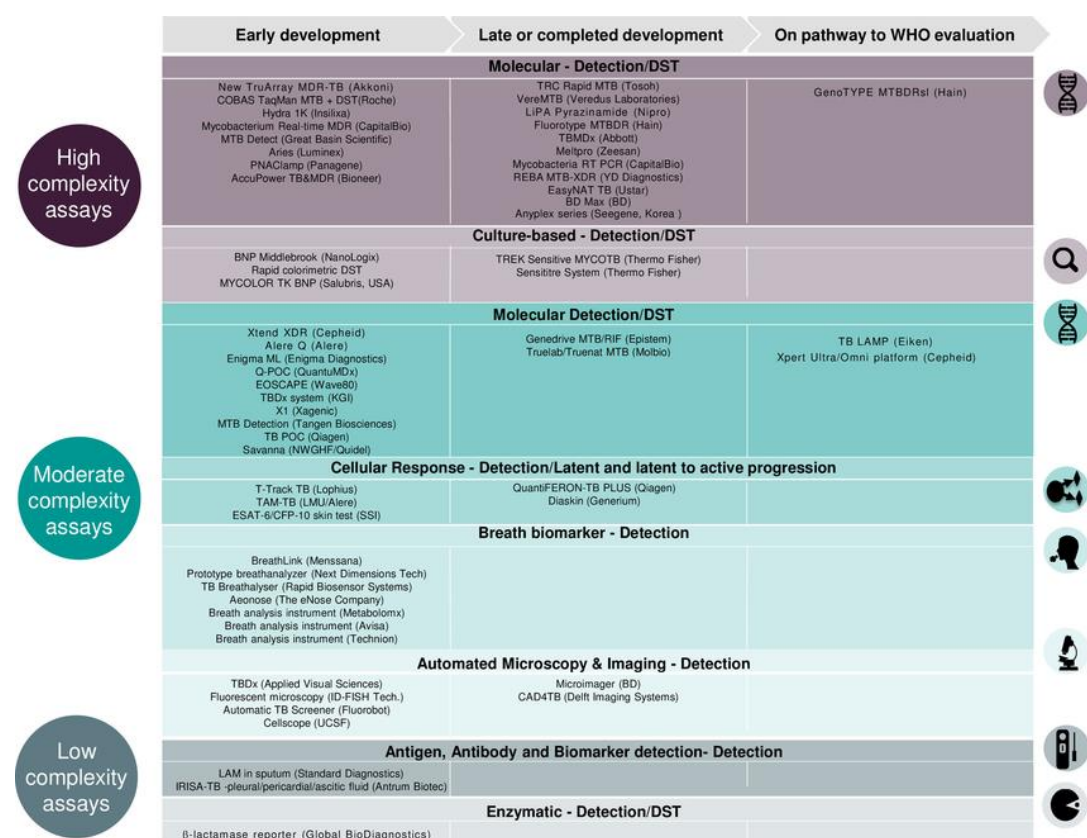


Figure 1.5 Current tuberculosis diagnostics pipeline listing the development phases and the types of technologies in development or evaluation

Reproduced from Tuberculosis diagnostics in 2015 by Pai et al. (94)

1.5.5.1 Smear microscopy

Smear microscopy of mycobacteria for TB diagnosis has been used for more than 100 years. The Ziehl-Neelsen (ZN) stain to examine the presence of acid fast bacilli in smear under light

microscopy is the most common method performed in almost all microbiology laboratories as the primary means of TB diagnostics. The principle of the method is based on resistance of mycobacteria to the decolourisation by acid alcohol rinse after bacteria are stained with phenol dyes. This feature is attributed to the mycolic acid component present in cell wall of mycobacteria. This method is simple and relatively rapid (30 minutes), but requires 100 microscopic field observations and cannot differentiate MTB from other non-tuberculous mycobacteria. The concentration of bacilli should be at least 5000 – 10,000 bacilli per millilitre of sputum to be visually detected (95). For diagnosis of adult PTB the sensitivity of microscopy was only approximately 20-40% for single sputum and 70% for two sputa (96, 97). The sensitivity of ZN smear is even lower in paediatric samples. In a recent review, the pooled value of sensitivity of ZN staining was found to be less than 10% on sputa (10, 98), and very poor on gastric aspirates (99) and other specimen types. The inefficiency of ZN in children could be explained by the paucibacillary nature of paediatric TB. In adult patients suspected of TB meningitis, concentration of high volumes of cerebrospinal fluid (CSF) does improve the yield of ZN considerably (from 28% to 72%) (100) and is successful in diagnosis of tuberculous meningitis. However, it is often not feasible to collect such high volumes of CSF in children (at least 4 ml).

Fluorescence microscopy (FM), using auramine to facilitate observation of bright acid-fast bacilli, can be performed at 400 times (rather than 1000) magnification which decreases reading time. The sensitivity of FM on sputum in adults was found to be on an average 10% more sensitive than conventional microscopy (95% CI: 5–15%) and almost 98% specific (9, 96, 101). However, FM only gained 2% of sensitivity compared to light microscopy performed on sputum in diagnosing TB in children (14.6% versus 12.5% , $p < 0.001$) (102).

In summary, although smear microscopy is commonly performed in the high burden countries to diagnose TB, current staining methods are rarely positive in paediatric TB cases (45) and other detection methods should be developed for paediatric TB.

1.5.5.2 Mycobacterial culture

Mycobacterial culture is considered the gold standard for confirmed diagnosis of TB, although sensitivity and specificity are not 100%. Isolation of MTB is performed on agar-based medium (solid culture) or liquid medium (liquid culture). The commonly used solid media are the egg-based Löwenstein-Jensen (LJ) or agar-based Middlebrook 7H10 media. The liquid media which are often used for the recovery of mycobacteria from clinical materials are based on Middlebrook 7H9. Liquid culture is more sensitive for MTB detection and also has shorter time to positivity; however, it is also more often contaminated, or cross-contaminated. The choice between these methods usually depends on the availability of resources.

In microbiology laboratories in resource-limited countries, LJ slants are the most commonly used solid medium because of lower cost and being easy-to-use (104). The major limitation of this method is the slow turnaround time of 4-6 weeks (105), which is too late to influence clinical decision-making. In addition, LJ has a lower yield in comparison to liquid culture in diagnosing TB (22.8% vs. 29.7%) (104, 106).

There are different commercial automatic liquid based culture systems for TB isolation such as MB/BacT [Biomérieux, Marcy l'Étoile, France], BACTEC 9000 [Becton Dickinson, NJ, USA] and the mycobacterial growth indicator tube [MGIT; Becton Dickinson]). MGIT culture has been endorsed by WHO since 2007 for early diagnosis of TB. The time to detection was reported to be 2 weeks shorter compared to LJ culture (107, 108). In a

paediatric study by Nhu *et al*, comparing MODS and GeneXpert MTB/RIF to the MGIT gold standard, the time to detection of MGIT was 8 days (IQR 7–13) for diagnosing TB in children (109). Most studies performed on specimens from PTB suspected patients showed that MGIT culture is more sensitive than solid culture (104). Although liquid culture has an advantage of higher sensitivity and shorter turnaround time in compared to solid culture, the wide implementation of automated liquid culture have been limited because of high costs in high burden countries with limited resources (104). Therefore, a lower cost alternative has been developed, known as microscopic observation drug susceptibility (MODS).

Microscopic observation drug susceptibility assay

MODS is a manual liquid culture system developed by a group in Peru for early detection of TB and determination of drug susceptibility (110). The principle of MODS is based on microscopic observation of MTB growth in cell culture plate wells containing Middlebrook 7H9 medium with and without antituberculous drugs. The MODS assay uses an inverted microscope and standard equipment that most basic microbiological laboratories will have, which is suitable for resource limited settings. MODS has the additional benefit that culture plates for identification of MTB and susceptibility can be sealed and read while sealed and therefore do not require additional biosafety measures. The morphology of MTB growth on MODS culture is illustrated in the Figure 1.6, showing characteristic cord formation. This is the formation of long, rope-like strings of bacilli in which the bacilli align along the long axis in chains. Although cord formation is often used for the presumptive identification of MTB over other non-tuberculous mycobacteria, in reality the sensitivity of the method depends not only on the experience of the technician but also on the number of bacilli present and therefore it is not an accurate technique for MTB identification (111).

Regarding identification of MTB, most evaluations of MODS for TB diagnosis have been performed in adults with pulmonary TB. In comparison with the MGIT commercial assay, MODS shows similar sensitivity and shorter or similar time to detection (10, 112-114). In comparison with solid culture (LJ), MODS has a higher sensitivity with shorter time to detection (115, 116). Limited number of studies showed performance of MODS for diagnosis of TB in children (10), which are discussed in more detail in chapter 3



Figure 1.6 MTB characteristic cording in MODS plate at $\times 400$ magnification with inverted microscope (117).

1.5.5.3 Nucleic acid amplification tests

The addition of molecular tests can improve the speed and sensitivity to detect active tuberculosis (118). The turnaround time obtained by nucleic acid amplification tests (NAATs) may be within hours rather than days. However, current available NAATs are not sufficiently sensitive for a negative result to exclude tuberculosis, especially in extra-pulmonary TB.

There are many commercial NAATs for TB in different stages of development (Figure 1.7). To be considered for paediatric TB, a commercial NAAT should demonstrate good sensitivity and specificity for adult PTB. The sensitivity for paediatric TB will be lower than for adult TB due to the paucibacillary nature of paediatric TB. Some of the more advanced NAATs, which have potential for TB diagnosis in children, are reviewed here. The only NAAT test to

have received WHO endorsement for diagnosis of paediatric TB is the GeneXpert MTB/RIF assay.

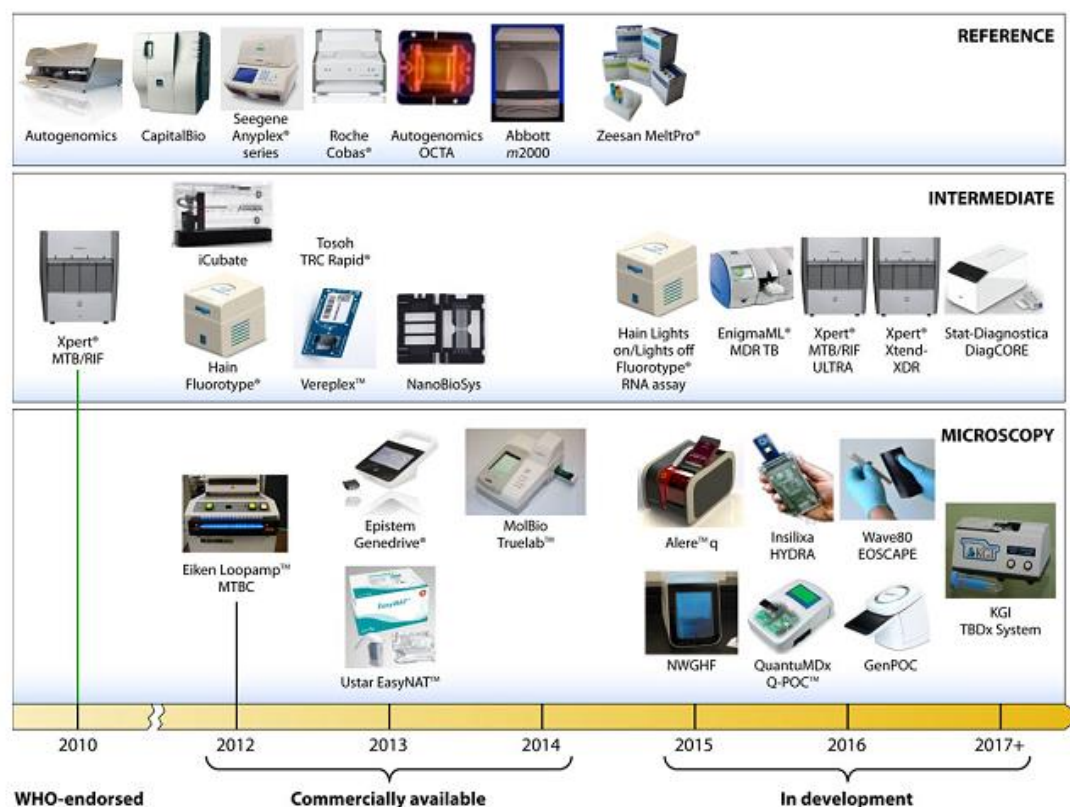


Figure 1.7 Pipeline of molecular diagnostics for tuberculosis, by level of deployment (i.e. reference, intermediate, and peripheral microscopy laboratories)

Reproduced from Tuberculosis diagnostics in 2015 by Pai et al. (94)

Loop-Mediated Isothermal Amplification assay

Loop-Mediated Isothermal Amplification assay is a manual TB detection method based on loop-mediated isothermal amplification (LAMP) developed by Eiken Chemicals, Japan. The assay is simple, rapid (15-40 min), operation only requires isothermal conditions (requiring only a heat block) and results can be read by the naked eye (119, 120). Those characteristics should facilitate implementation in laboratories in high burden countries. The initial evaluation of LAMP in high burden countries was disappointing, however, with low specificity, and LAMP is currently being re-optimised. The recent upgraded LAMP version, PURE-LAMP, performed well on smear positive sputum (sensitivity of 89%) but only fair on smear negative sputum (sensitivity of 54%) for MTB detection using solid culture as the reference method in a study among adults in China (121). Larger multi-country studies will be needed to demonstrate sufficient accuracy for endorsement by WHO and scale-up. There is no evidence regarding sensitivity of the LAMP test for diagnosing TB in children (122).

GeneXpert MTB/RIF

GeneXpert MTB/RIF (Cepheid, USA) is a molecular assay, which can simultaneously detect MTB and rifampicin resistance, generating a result within 2 hours. This assay was endorsed by WHO in 2010 and was recommended for TB diagnosis in children since 2013 (123) (124). The accuracy of GeneXpert MTB/RIF for diagnosing TB in children was extensively investigated in various studies (98, 109, 125, 126). For diagnosis of PTB in children, the overall estimated sensitivity of a single GeneXpert test was 62% (95% CI: 51 - 73 %) for expectorated or induced sputum, 66% (95% CI: 51 - 81 %) for gastric fluid, when compared with culture as reference method (solid and liquid culture) (127). For diagnosing extra-pulmonary TB in children, limited evidence of GeneXpert MTB/RIF performance on non-respiratory specimens showed lower sensitivity compared to culture. GeneXpert MTB/RIF performed on CSFs showed sensitivity of 39% against culture for diagnosing paediatric

tuberculous meningitis in a study in Cape Town, South Africa (128). Available evidence from adult studies also showed the suboptimal sensitivity of GeneXpert MTB/RIF for EPTB diagnosis in comparison to liquid culture (pleural TB: 21.4%, 95% CI [8.8–33.9%]; TB meningitis: 62.8%, 95% CI [47.7–75.8%])(129)

The major limitation of GeneXpert MTB/RIF is the high cost and test availability, which varies widely by country. South Africa has implemented GeneXpert MTB/RIF nationwide (130), while most other countries have placed GeneXpert MTB/RIF at a few key locations including centralised or reference TB laboratories. Priority is normally given to patients with presumed drug resistance or HIV infection, due to limited test availability and a focus on detection of MDR-TB. The majority of children with TB initially present at clinics or general hospitals, where the GeneXpert MTB/RIF assay is rarely available (123).

1.5.5.4 Serodiagnostic tests

Serodiagnostic tests, which detect antigen or antibody, are highly desirable for TB, due to potential speed and ease of use. However, accurate serodiagnostic tests have proved elusive for TB (131). Detection of MTB lipoarabinomannan (LAM) cell wall antigen in urine for TB diagnosis has been developed as a point of care test because it generates rapid results. LAM is present in the urine of TB patients. Early studies in adults showed the test has a low sensitivity but high specificity, and demonstrated the usefulness in ruling in TB in the group of patients with advanced HIV with low CD4 cell counts (132, 133). However, the test showed a very poor performance in children, even in children with disseminated (miliary) tuberculosis (134-136). In a prospective study of 535 children with suspected PTB, the LAM test showed a sensitivity of 48.3% and specificity of 60.8% against culture and did not have additional yield in HIV infected children. The LAM urine test also showed the little value in

diagnosis of tuberculous meningitis in children with sensitivity of 4.8% and a specificity of 93.1% against the clinical diagnosis (137).

1.5.5.5 Biomarkers

A biomarker can be defined as a molecule found in blood, other body fluids, or tissues which can be used as a measurable indicator of normal biological processes. During recent years, searching for TB related biomarker has been an active area of research, in particular biomarkers which are able to distinguish between active TB, latent TB infection, and no disease (138). Availability of an accurate biomarker for TB may be a useful tool to improve the diagnosis of TB in children (139), because the current diagnostic tests are limited by the paucibacillary nature of paediatric TB as compared to the adult. However, the research into biomarkers in children diagnosis has been limited. The validation of biomarkers for use in TB diagnostics in children is also challenging due to the lack of standard sets of samples from children with and without (active or latent) tuberculosis.

1.5.5.6 Interferon gamma release assays

Interferon gamma release assays (IGRA) were developed as an alternative to the tuberculin skin test (140). Whole blood or peripheral blood mononuclear cells are stimulated *in vitro* with antigens from MTB and interferon gamma is measured. Early IGRA, which uses ESAT-6 and CFP-10 antigens for stimulation, are not confounded by prior BCG vaccination because these antigens are not found in the BCG vaccine strains. Two commercial IGRAs are currently FDA approved for the diagnosis of MTB infection: The QuantiFERON-TB Gold In-Tube test (Cellestis Limited, Carnegie, Victoria, Australia) and T-Spot test (Oxford Immunotec, Abingdon, United Kingdom). However, the use of IGRA for diagnosis of disease in children is not recommended, because IGRA cannot distinguish between latent and active TB disease, in particular in endemic settings where the latent infection is highly prevalent (141, 142).

Table 1.4 Laboratory tests for diagnosis of active TB and drug resistance

Details of policy guidance at: <http://www.who.int/tb/laboratory/en/>

Reproduced from Heemskerk *et al* 2015 (45)

Diagnostic tests for active TB					
Test type	Principal commercial tests	WHO policy	Advantages	Limitations	
Smear microscopy	Non-commercial	Recommended	Inexpensive, simple, rapid, specific	Cannot differentiate NTM ^a and MTB	
LED microscopy		Recommended	Inexpensive, simple, rapid	Cannot differentiate NTM ^a and MTB	
Automated real-time nucleic acid amplification	GeneXpert MTB/RIF	Recommended	Rapid (2 h to result). Detects smear-negative TB. Also detects RIF resistance	Higher cost than smear	
Loop-mediated isothermal amplification test kit for TB	LAMP assay	Not recommended. Under further development	Rapid, simple	Subjective interpretation and poor specificity	
Rapid speciation strip technology		Recommended	For rapid differentiation of NTM ^a and MTB	Expensive	
Serodiagnostic tests	Over 20 commercial variants	Not recommended		Poor sensitivity and specificity	
Interferon-Gamma release assays	QuantiFERON-TB Gold In-Tube test, T-Spot test	Not recommended for diagnosis of active TB		Complex to perform and indeterminate results relatively common	
Drug susceptibility tests					
Test type	Principal	WHO policy	Drugs tested	Advantages	Limitations
Phenotypic DST on solid or liquid media	Non-commercial	Recommended for USE	All drugs ^b	Gold-standard	Extremely long time to result (6–12 weeks)
Commercial liquid culture and DST systems	Bactec MGIT	Recommended for USE	STR, INH, RIF, EMB, PZA	Faster than solid culture media. Ten days if direct testing	Expensive

Line probe assay first-line	MTBDR-Plus; INNO LiPA-RIF TB	Recommended for USE on smear-positive samples	RIF, INH	Result in 2 days	Expensive
Line probe assay second-line	MTBDRsl	Not yet recommended due to insufficient evidence	Fluoroquinolones, aminoglycosides and EMB	Result in 2 days	Low sensitivity for ethambutol
Automated real-time nucleic acid amplification	GeneXpert MTB/RIF	Recommended for USE	RIF	Result in 2 h	Cartridge price reductions only available in low middle income countries
Microscopic observation drug susceptibility (MODS)	Non-commercial	Recommended for USE	RIF, INH	Low-tech. 10–14 days for result	Subjective interpretation. Laborious manual plate reading ^c
Colometric redox indicator (CRI)	Non-commercial	Not yet recommended due to insufficient evidence	RIF, INH	Low-tech. 10–14 days for result	Subjective interpretation
Nitrate reductase assays (NRA)	Non-commercial	Not yet recommended due to insufficient evidence	RIF, INH	Low-tech 10–14 days for result	Subjective interpretation
Phage assays	FASTplaque, luciferase reporter phage assay	Not recommended	RIF, INH	N/A	Poor specificity
Sequencing	Non-commercial	No policy	Depends on gene regions sequenced	Can provide information on multiple drugs simultaneously	Requires specialist interpretation. Not generally available outside research centres

^a NTM: non-tuberculous mycobacteria; ^b Reliable for first-line drugs (except pyrazinamide), fluoroquinolones and aminoglycosides. Second-line DST should be interpreted in context of treatment history and local prevalence of resistance (if known); ^c Indicator well must be incorporated to differentiate NTM from MTB

1.5.6 Treatment of tuberculosis

1.5.6.1 Purpose of treatment

The main goals of TB treatment are to cure the patient of TB, to prevent death from TB or its late effects and to prevent TB relapse. Successful TB treatment also prevents further transmission of TB and drug resistance development. The principles of treatment of TB in children are the same as for the treatment of TB in adults (143). TB treatment needs to be taken at least six months with a combination of TB drugs with different actions. It consists of two phases: an intensive phase followed by a continuation phase. In the first phase, the TB drugs rapidly eliminate the majority of actively replicating MTB bacteria. The continuation phase aims to eradicate any remaining dormant or near-dormant bacteria while preventing the emergence of drug-resistant organisms. The treatment regimen of susceptible TB in children will be discussed in the next section.

1.5.6.2 Treatment regimens

The first-line anti-TB agents form the core of treatment regimens. They include: isoniazid (INH), rifampicin (RIF), ethambutol (EMB), pyrazinamide (PZA) and streptomycin (SM). The standard treatment regimen for paediatric TB in the area with high HIV prevalence or high isoniazid resistance or both is an intensive phase of isoniazid (H), rifampicin (R), ethambutol (E) and pyrazinamide (Z), followed by two months of isoniazid and rifampicin (usually written as 2HRZE/4HR). The three-drug antituberculous regimen (2RHZ/4RH), which was previously recommended for less severe cases of childhood TB, is no longer recommended by WHO for any form of childhood tuberculosis since 2009, due to high likelihood of undetected drug resistance (144)

Recommended treatment regimens by WHO are summarised in adolescents and older children (once they reach a body weight of 25 kg) can be treated at adult dosages. Prior to

2006, paediatric TB doses were extrapolated from adult doses. However, following a series of pharmacokinetic studies in children, mainly from South Africa, it was concluded that children were severely under-dosed using these guidelines. This is due to a more rapid drug metabolism in children and a higher surface area:volume ratio in young children; a well recognised phenomena in paediatrics. In 2010, a systematic review of available data showed no safety concerns and the WHO dose guidelines were therefore revised to increase the paediatric dose of all drugs, except streptomycin.

Table 1.5 Revised WHO recommendations for first-line drug dosage in children showing the 2006 and 2010 recommendations.

Drug	Daily dosage (dose range) in mg/kg 2010 recommendations (143, 145)	Daily dosage (dose range) in mg/kg 2006 recommendations (146, 147)
INH	10 (7-15)*	5 (4-6)
RIF	15 (10-20)	10 (8-12)
PZA	35 (30-40)	25 (20-30)
EMB**	20(15-25)	15 (15-20)*
SM	15 (12-18)	15 (12-18)

* The higher end of the range for isoniazid dose applies to younger children; as the children grow older the lower end of the dosing range become more appropriate

**Previously, EMB was not recommended for children less than 5 years old due to potential ocular toxicity but this was withdrawn in the 2010 guidelines following a review of the evidence.

1.6 Tuberculosis resistance

1.6.1 Classification of tuberculosis resistance

According to WHO definitions, drug resistant TB (DR-TB) is classified into 4 discrete categories: mono resistant, poly resistant, multidrug resistant and extensively drug resistant.

In addition, the classification is based on the history of treatment into 2 categories: acquired and primary drug resistant TB. Patients are defined as having primary drug resistance if there is no history of previous TB treatment when resistance is diagnosed. Patients are defined as having acquired drug resistance if they develop drug resistance while on TB treatment or are diagnosed with drug resistant MTB that is genetically identical to isolates from the same patient collected before treatment or during a previous episode of TB. In 2011, the definition of drug resistance in children was proposed in the first consensus statement on drug resistant tuberculosis in children (Table 1.6)

Primary drug resistance occurs when a person becomes infected with drug-resistant MTB, transmitted from an infected donor, and develops active TB. Acquired drug resistance happens when individuals develop resistance during treatment, which is thought to be caused by poor treatment quality leading to the selection and stepwise amplification of mutant resistant MTB. Specific mutations associated with drug resistance are described in more detail in the next sections. Factors that contribute to poor treatment outcomes include: (i) inappropriate treatment due to lack of treatment guidelines and training, poor patient education and treatment support, poor management of treatment response and adverse drug reactions, (ii) inadequate drug quality and supply due to stocks running out, low quality or counterfeit drugs and poor storage conditions, (iii) incomplete adherence to treatment by the patient. People who belong to vulnerable groups (HIV infection, drug abuse, alcoholism) or have poor socioeconomic conditions have poor access to health care service generally have a higher risk of drug resistance development (148).

Table 1.6 Terminology for Drug-Resistant Tuberculosis in Children (149)

Terms of drug resistance categories	Definition
Monoresistant	Resistance to a single TB drug
Polyresistant	Resistance to 2 or more TB drugs other than both rifampicin and isoniazid
MDR-TB	Resistant to at least both rifampicin and isoniazid
Pre-XDR	MDR-TB with resistance to either a fluoroquinolone, or at least 1 of 3 injectable second-line TB drugs, but not both
XDR-TB	MDR-TB with resistance to both a fluoroquinolone and at least 1 of 3 injectable second-line TB drugs
Primary resistance	DR-TB that results from transmission of DR MTB. This could be any of the after clinical situations in a child newly diagnosed with confirmed or probable DR-TB:
	(a) never treated: a child without previous TB treatment who has not yet received any TB treatment; or
	(b) previously treated: a child who was previously treated with first-line drugs who was either cured or completed that treatment regimen; or
	(c) Currently receiving treatment: a child who is receiving first-line drugs for presumed drug susceptible-TB disease (DS-TB).
Acquired resistance	A child previously diagnosed with confirmed DS-TB disease who developed DR-TB disease (or resistance to additional drugs) during TB treatment

1.6.2 Transmission of drug resistance

Transmission of drug resistant TB in populations is thought to be attributable to both socioeconomical factors and intrinsic microbiologic factors. The socioeconomical factors that facilitate TB transmission include poor ventilation, crowding, poor infection control measures

(especially in closed settings). It has long been hypothesised that MDR-TB is less-transmissible than drug susceptible TB, due to fitness costs of resistance. However, it has now been shown that MTB can develop compensatory mechanisms to mitigate the fitness costs of resistance and some MDR TB strains may be as transmissible as drug susceptible strains (150, 151). Reports have also suggested that certain strains of the Beijing lineage carry a lower fitness cost for certain mutations than other lineages. The reasons for this are not completely known and may be due to mechanisms such as efflux pumps or other compensatory mechanisms such as higher tolerance for oxidative stress that was observed in the INH resistant strains carrying *katG* mutations (152-154). Catalase peroxidase encoded by *katG* also functions to protect against oxidative stress. It was reported that overexpression of *ahpC* encoding for alkyl hydroperoxidase reductase in *katG* mutants may compensate for loss of *katG* activity. In the epidemiological study by Buu *et al*, MTB of the Beijing genotype resistant to streptomycin showed higher rates of transmission than other genotypes (155).

1.6.3 Introduction of first line anti TB drugs

The first line drugs include rifampicin, isoniazid, ethambutol and pyrazinamide. Illustration of the targets of these anti-TB drugs are shown in Figure 1.8.

Rifampicin (RIF) was introduced as TB treatment in 1967. RIF prevents RNA transcription of MTB by inhibiting the beta subunit of RNA polymerase. Consequently, protein synthesis is interrupted, causing bacterial death (156).

Isoniazid (isonicotinic acid hydrazide, INH) has been the most commonly used antituberculous drug since recognition of its clinical activity in 1952. INH is a very powerful anti-TB drug with a very complex mechanisms of action against mycobacteria (157). INH is a pro-drug which requires to be activated by the MTB catalase-peroxidase enzyme KatG (158). The activated INH has several targets in mycobacteria. It interferes with enzymes involved in cell wall mycolic acid synthesis (159) and also metabolism of DNA and lipids.

Ethambutol (EMB), discovered in 1961, inhibits MTB cell wall synthesis via the inhibition of biosynthesis of arabinogalactan, a major polysaccharide in the MTB cell wall. This activity interrupts the growth and replication of MTB, therefore EMB is only active against replicating bacilli (131).

Pyrazinamide (PZA) was first tested as an anti-TB drug in the early 1950s. Similar to INH, PZA is a pro-drug, which only has antibacterial activity after activated by the enzyme pyrazinamidase (PZase) (160). Its active form, pyrazinoic acid (POA), targets the cell membrane resulting in dysfunction of ion exchange membrane channels, causing an intracellular pH change. This change disrupts enzyme activities and results in cell death. Uptake and intrabacillary accumulation of POA is enhanced when the extracellular pH is acidic (161). PZA has been hypothesised to act against bacilli residing in acidified compartments of the lung that are present during the early inflammatory stages of infection (162).

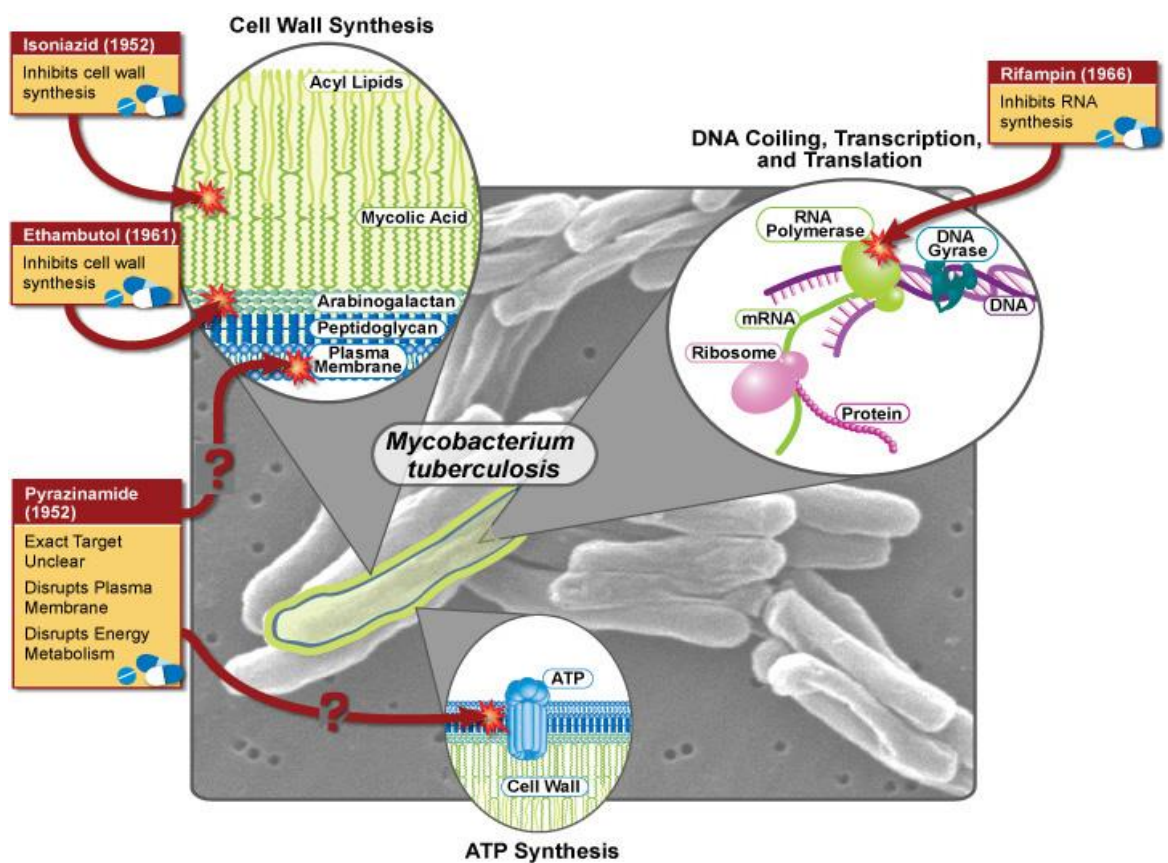


Figure 1.8 Illustration of the targets of first line anti-TB drugs.

(Source: <http://www.niaid.nih.gov>)

1.6.4 Molecular mechanism of drug resistance to the first line anti TB drugs

Resistance to anti-TB drugs is caused by spontaneous mutations in the bacterial genome (as opposed to plasmids and transferable elements). The rate of mutation varies by drug between 1 in 10^6 - 10^9 per cell division. Therefore, a small number of drug resistant bacilli to any given drug will be present in a large MTB populations (e.g. in granulomas), regardless of drug pressure. Therefore, anti-TB drugs have to be given in combination, as the risk of a mutant containing two resistance mutations is $<10^{-18}$. If monotherapy is given, the resistant population will be selected, and emerge as dominant. The risk of mutation may also be related to the genetic background of the MTB (163). For example, several epidemiological studies have shown association between specific mutations and the Beijing lineage of MTB, while *in vitro* work has suggested this may be due to a higher spontaneous mutation rate or ability to develop compensatory mechanisms to ameliorate the fitness costs (150, 151, 164, 165). MTB does not support plasmids and therefore does not acquire drug resistance through plasmid transfer. Resistance may also occur through up-regulation of efflux-pump mechanisms but these are poorly understood in MTB at the present time. In the following sections, I summarise the molecular mechanisms of drug resistance to individual first line drugs.

1.6.4.1 Isoniazid

INH is the most commonly used antituberculous drug and is also used as monotherapy for latent TB, therefore, resistance to INH occurs more frequently among clinical isolates than other agents (166). INH resistance occurs most often due to mutations in *katG* (the gene encoding the mycobacterial catalase-peroxidase KatG), occurring in 50–80% of INH-resistant isolates. INH is a pro-drug which requires KatG to be activated. Mutations, particularly at the 315 codon, reduce the ability of KatG to activate INH (167). A single point mutation resulting in substitution of threonine for serine at residue 315 (S315T) accounts for the majority of high-level INH resistance among clinical isolates. Point mutations at other sites in *katG* and

deletion of sections or the whole gene may also confer resistance. INH resistance may also arise from mutations in *inhA*, resulting in reduced affinity of the enzyme for NADH without affecting its enoyl reductase activity or in the promoter region of the *mabA-inhA* operon (most commonly -15 C-T) resulting in overexpression of the wild-type enzyme. *inhA* mutations generally confer 'low-level' INH resistance (1-2µg/ml) while *katG* mutations often confer much higher level resistance (>3µg/ml). As opposed to other INH resistance conferring mutations, the *katG* 315 mutation also does not come with a fitness cost. Approximately 10-20% of phenotypically INH resistant isolates carry no mutations in known resistance conferring genes. This is one of the reasons for the current limitations of NAATs, such as the line probe assays, for detecting INH resistance.

1.6.4.2 Rifampicin

Resistance to RIF in MTB results from single point mutations in the 81 base-pair rifampicin-resistance-determining region of *rpoB*, which encodes a subunit of the mycobacterial RNA polymerase (168). Mutations in codons 531 [Ser] and 526 [His] are the most common. Approximately <5% do not contain any mutations in the *rpoB* gene, suggesting alternative resistance mechanisms, potentially including altered rifampicin permeability or mutations in other RNA polymerase subunits (169). However, this may be due to inaccurate phenotypic testing or misidentification of isolates as rifampicin resistant. Recent work has attempted to elucidate the differences in identification of rifampicin resistant isolates between phenotypic DST methods, especially solid vs. liquid media. Resistance to rifampicin alone is rare, and more than 90% of rifampicin-resistant isolates are also resistant to INH. Rifampicin monoresistance is more commonly identified in people living with HIV, but remains rare. Therefore, rifampicin resistance is used as a surrogate marker for MDR-TB (170).

1.6.4.3 Pyrazinamide

PZA resistance has been attributed primarily to mutations in *pncA* encoding pyrazinamidase, which activates the pro-drug PZA into its active metabolite (171). Most mutations, including point mutations, deletions, and insertions, have been reported in a 561-bp region of the open reading frame or in an 82-bp region of its putative promoter. A small percentage of isolates with high-level PZA resistance contain no mutations in *pncA* or its promoter, suggesting alternative mechanisms of resistance such as deficient uptake, enhanced efflux, or altered *pncA* regulation.

1.6.4.4 Ethambutol

Resistance to EMB in MTB is usually associated with point mutations in the *embCAB* operon (172). As the majority of EMB-resistant clinical isolates contain mutations in *embB*, EmbB is considered to be the main target of EMB (173). Line probe assays targeting *embB* mutations have poor sensitivity (approximately 60%) and 25% of EMB resistant isolates have no mutation in the *embCAB* operon, therefore other mechanisms of resistance clearly remain to be identified.

Molecular mechanisms of drug resistance in MTB were thoroughly reviewed by Almeida *et al.* (174). In the CRYPTIC study (Comprehensive Resistance Prediction for Tuberculosis: an International Consortium), funded by the Wellcome Trust and led by the University of Oxford, 100,000 MTB genomes from around the world will be sequenced, aiming to definitively identify all drug-resistance associated mutations and ultimately improve the molecular identification of drug resistance in MTB (<http://modmedmicro.nsms.ox.ac.uk/cryptic/>).

1.6.5 Drug resistance epidemiology in children

The emergence and spread of drug resistance in MTB has posed a serious threat to global TB control. Globally, the WHO reports an estimated prevalence of 3.6% and 20.2% among

notified TB cases for primary and acquired multidrug resistant tuberculosis (resistant to both rifampicin and isoniazid, MDR-TB), respectively, with significant country and regional variations. Children are not a priority in national surveillance programmes and the difficulty of isolating MTB from children means drug susceptibility is rarely confirmed. In fact, children (defined by WHO as individuals less than 15 years old) are excluded in most designs for national TB surveys or, if included, the percentage of children is insignificantly small. For instance, in the Global Drug-Resistant Surveillance Project surveys, children account for less than 2% of the more than 300,000 patients (175). Therefore, the estimated number of drug resistant TB in children can only be inferred from adult data.

With up to 20% of the TB caseload found in children (<15 years of age) in high-burden settings (23, 52), the number of children with drug-resistant (DR) TB and MDR-TB is likely to be substantial (Table 1.7), as was described e.g. in a recent review by Yuen *et al*, with risk variation by geographical setting, depending on the background prevalence of INH-resistant MTB circulating in the population (Figure 1.9). It was estimated that 12.1% of all children with TB had isoniazid-resistant tuberculosis, representing about 120,000 incident cases of isoniazid-resistant tuberculosis in children in 2010, with the majority of these found in the Western Pacific and South-East Asian regions (176, 177). Globally, the number of children with MDR-TB is estimated at 3.2 % of all paediatric TB cases, which would give 32,000 cases of paediatric MDR-TB occurring in 2010 (178) (Table 1.7). MDR-TB in children is thought to be usually transmitted from an MDR-TB infected adult, usually a parent or caregiver, rather than secondary resistance development within the child because of the paucibacillary nature of TB in children. The limited published information about children with MDR-TB is an obstacle to efforts to advocate for better diagnostics and treatment. However, the scattered nature of cases and difficulty of isolating bacteria to confirm the diagnosis makes clinical trials for MDR-TB in children extremely challenging.

Table 1.7 Estimated numbers of incident cases of TB disease and MDR-TB disease in children by region, 2010 (178)

WHO region*	Estimated number of child TB cases			Estimated number of child MDR-TB cases		
	Estimate	95% Lower Confidence Bound	95% Upper Confidence Bound	Estimate	95% Lower Confidence Bound	95% Upper Confidence Bound
African region	279,825	250,187	308,717	4,736	2,829	6,848
Eastern Mediterranean region	71,162	60,320	83,193	2,417	339	5,087
European region	43,224	39,572	47,242	5,645	4,206	7,463
Region of the Americas	27,199	24,935	29,635	606	374	854
Southeast Asia region	397,040	350,615	447,474	10,000	4,993	15,568
Western Pacific region	179,515	159,246	202,626	8,349	5,639	11,610
Total	999,792	937,877	1,055,414	31,948	25,594	38,663

*These regions are aligned with those as defined by the World Health Organization (WHO)

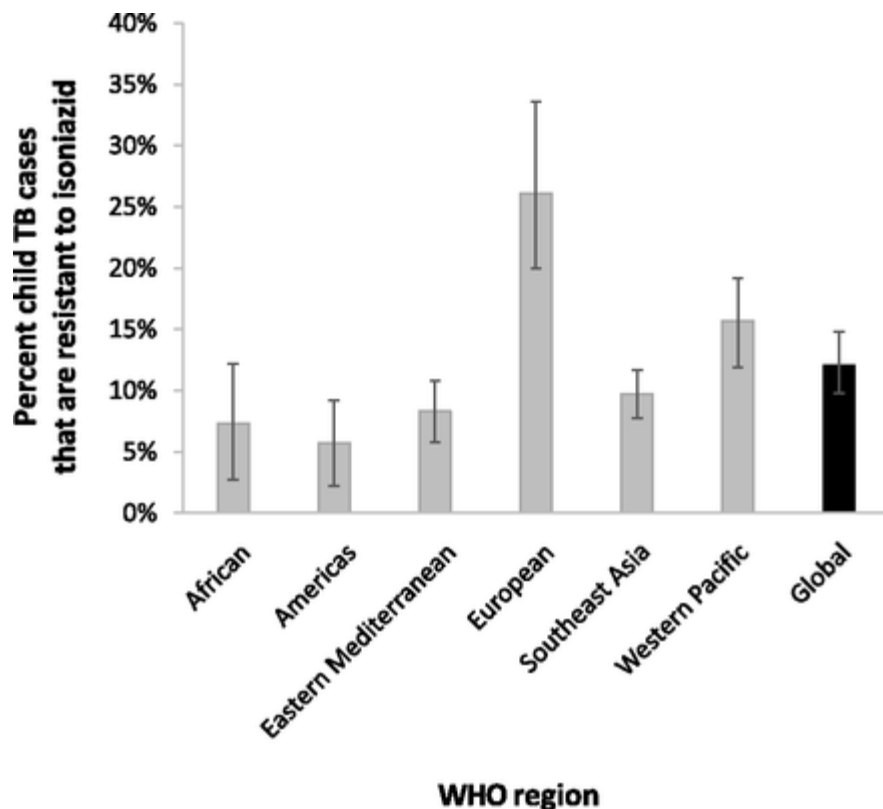


Figure 1.9 Estimated percentage of child TB cases that are resistant to isoniazid by region (177)

1.6.6 Drug resistance in Vietnam

Vietnam is ranked 12th among 22 high burden countries with TB and 14th among 27 countries with a high burden of MDR-TB, with burden being defined as an absolute numbers of cases. The estimated incidence and the estimated prevalence of TB in Vietnam is 144 per 100,000 population per year and 209 cases per 100,000 population, respectively (1). The fourth national drug resistance survey was conducted in 2011 (179). There are estimated to be about 5,100 MDR-TB cases among notified TB cases per year. The proportion of TB cases with MDR-TB among new and retreatment cases is estimated to be 4% and 23%, respectively, with around 6% among TB cases co-infected with HIV. Figure 1.10 shows the estimated number of MDR-TB patients among notified TB cases through Vietnam in 2013.

In another study in Hanoi, which investigated the status of drug resistance among newly diagnosed patients with sputum smear- and culture-positive TB, isoniazid and streptomycin resistance was detected in more than a quarter of TB patients (180). Drug resistance is slightly higher in southern Vietnam and is also higher in cities compared to rural areas, a pattern seen in many high burden countries (181).

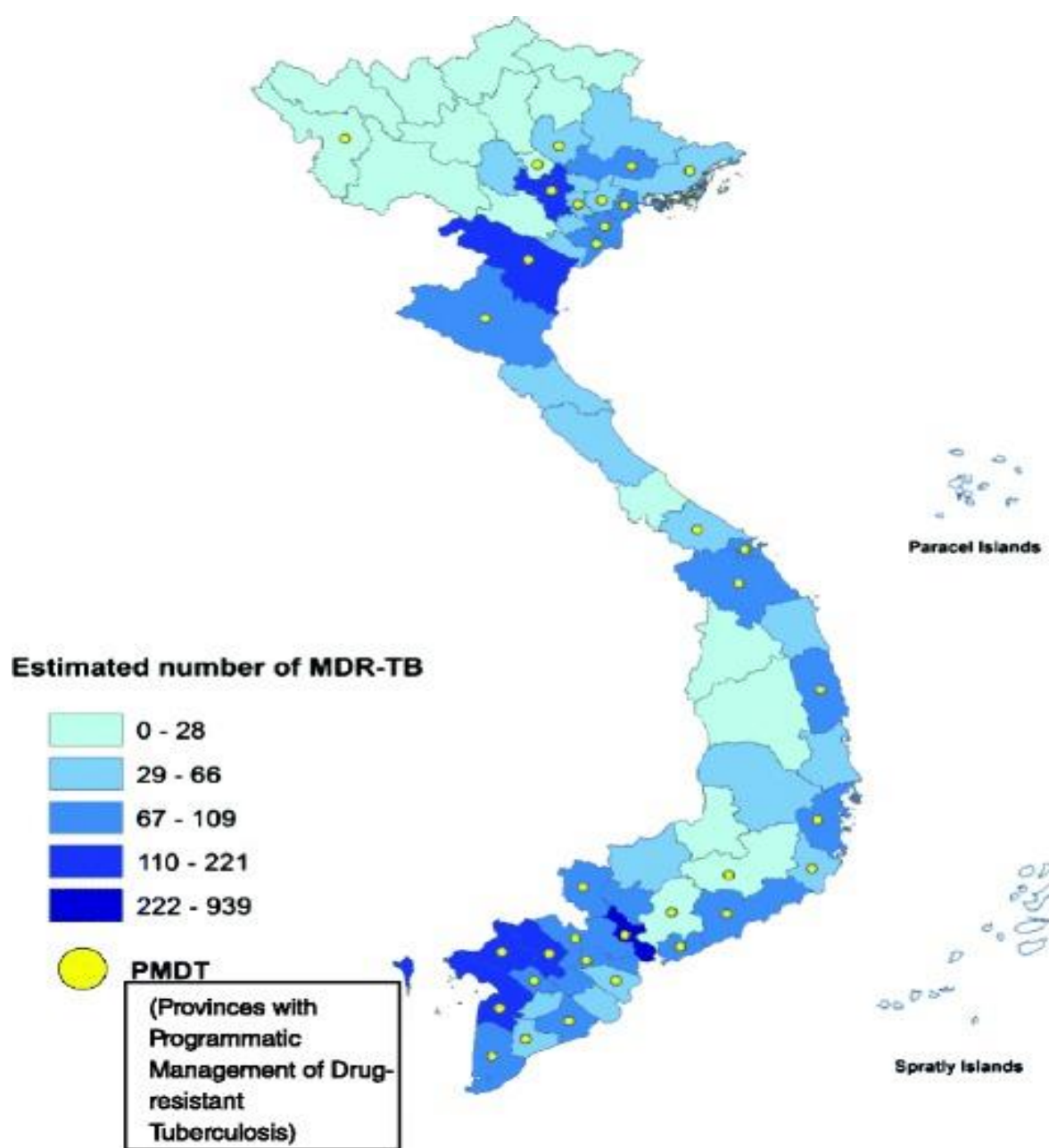


Figure 1.10 Estimated number of MDR-TB patients among notified TB cases in Vietnam, 2013 (165).

Children aged less than 5 years old account for 24.3% of the population (22 million children, population statistics 2014) (182). It was estimated that 351,000 children are infected with TB annually (National Tuberculosis Programme [NTP] survey 2006) (183), with 13,000 (5%-10%) children estimated to develop TB, but only a small number were reported by the NTP (6%). Besides the difficulties in diagnosis of TB in children as mentioned above, additional explainable factors causing the low notification case number among children in other high burden countries like Vietnam are ineffective case finding strategies (184), incomplete recording, imperfect reporting of current national TB control program (185). Although active household contact screening is recommended for improved case finding, it is rarely performed in Vietnam and most children are not included in active contact investigations (185, 186). For a long time, children have been excluded in national surveys of drug resistance. As a consequence, there is limited data of TB drug resistance among children in Vietnam. In line with WHO recommendations, the NTP has now begun to systematically collect data on paediatric TB and this will provide a baseline to improve both data and management of paediatric TB in Vietnam.

1.7 Molecular epidemiology of TB

1.7.1 Genetic diversity

Understanding the genetic epidemiology of a pathogen will help us to understand transmission patterns within and between populations (including the spread of drug resistance), virulence and evolution of an organism. Early work on MTB genetic variation focused on isolates from Europe and North America and therefore the genetic diversity of global MTB was underestimated until the last 2 decades, when researchers started to systematically analyse global collections(187, 188). However, MTB is relatively clonal compared to other bacteria as evolution does not occur via horizontal gene transfer due to its

intracellular replication. Variation in MTB therefore principally arises from genomic deletions, rearrangements and point mutations. (189).

MTB has been classified into six major lineages. Initially this classification was based on large genomic deletions, but this classification has since also been confirmed by whole genome sequencing (190, 191). Each lineage is strongly associated with a geographic region and therefore the lineages may be known by either numbers or the name denoting the geographical area where they are predominantly isolated (188) (Figure 1.11). Lineage 4 is mostly found in Europe and America; while lineage 2 is predominant in East-Asia and lineage 3 in the Indo-Oceanic region. Lineages 5 and 6 are predominantly found in West Africa. A seventh lineage has recently been described from Ethiopia (34). Figure 1.11 shows the geographic distribution of the six lineages.

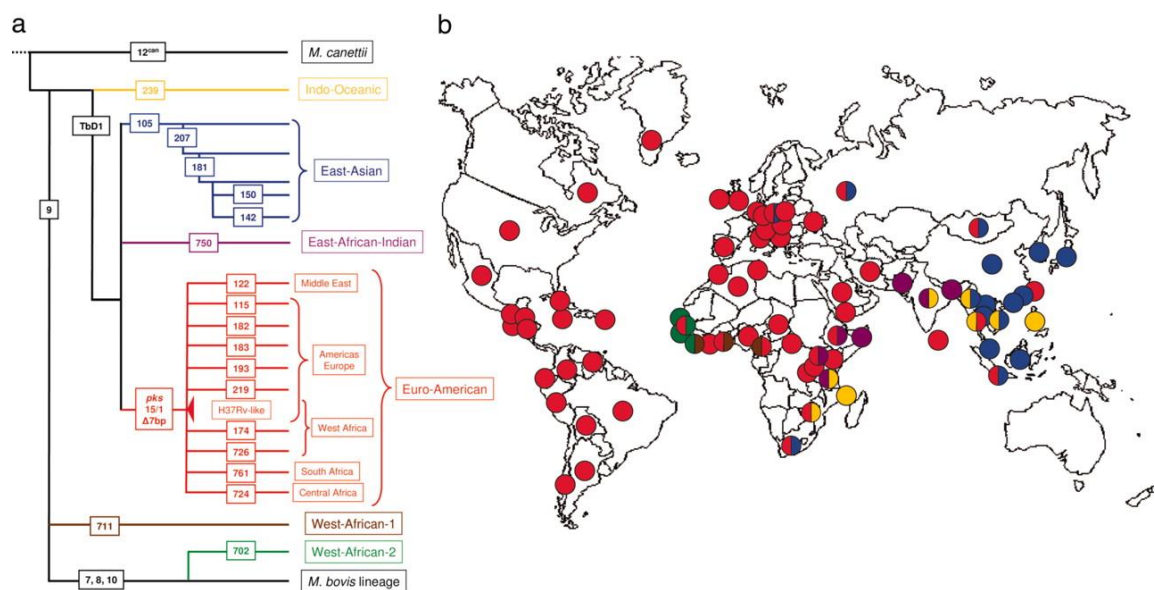


Figure 1.11 The global population structure and geographical distribution of MTB

(a) Six lineages of global phylogeny for MTB are defined by large sequence polymorphism (LPS). The names of the lineage-defining LSPs are shown in rectangles. The geographic regions associated with specific lineages are indicated. (b) The geographic distribution of six lineages (190).

Typing methods used in epidemiological studies for MTB are described in more detail below. Each method has advantages and disadvantages and the most appropriate method will depend on the geographical origin of the strains, as the discriminatory power of different typing techniques varies widely between the major lineages. The gold standard technique is now whole genome sequence analysis, and while this has become cheaper and more accessible in recent years, it remains beyond the reach of most national TB control programmes.

1.7.2 Genotyping method for MTB

1.7.2.1 IS6110 RFLP

IS6110 is the insertion sequence which was first recognised by Thierry *et al.* in the 1990s (192). This sequence is a 1,355 base pair long sequence, which is found within the MTB complex only. It is present at multiple copies in most members of the MTB complex, ranging from 0 to 25 copies. The varying copy number and location in the genome which can be visualised using probe hybridisation to digestion fragments is the basis of IS6110 genotyping of MTB.

The IS6110 restriction fragment length polymorphism (IS6110-RFLP) is a method starting with endonuclease digestion of the genomic DNA with the PvuII restriction enzyme, followed by separation of restriction fragments through gel electrophoresis, transfer onto a membrane and hybridisation with a labeled probe complementary to the sequence of IS6110 (193). Each fragment represents a single copy of IS6110 surrounded by flanking DNA of different lengths.

The IS6110-RFLP technique has been standardised. The method is highly discriminatory and reproducible. It was one of the most commonly used methods for MTB typing in the 1990s

and was long considered the gold standard in the molecular epidemiological investigation of TB. However, this technique has some major limitations and has now been largely replaced by simpler techniques. Firstly, the technique requires a large volume of high quality DNA for restriction enzyme digestion, which necessitates a subculture step. Secondly, it takes several days to perform and there is the need for sophisticated and expensive computer software for analysis and matching as well as experienced technicians. Finally, the discriminatory power is insufficient for those isolates with a low copy number of IS6110 (less than 5 copies) that circulate widely in Asia (194).

1.7.2.2 Spoligotyping

Spoligotyping (Spacer Oligonucleotide Typing) is based on identifying polymorphisms in the spacer units in the direct repeat (DR) region of the genome. The DR region comprises multiple identical 36bp regions interspersed with non-repetitive spacer sequences of similar sizes (195). Variation in this regions is usually caused by homologous recombination between adjacent or distinct DRs or by transformation due to the insertion of IS6110. Forty-three spacers among 60 are selected as a basis of standard spoligotyping. The presence or absence of these spacers is detected by PCR amplification of short sections of the region by using primers targeted to the direct repeat sequence. The resulting PCR fragments, which also contain the non-repetitive spacer sequences, are then reversely hybridised to immobilised probes for each of the 43 spacer sequences (196). The presence or absence of the spacers in each tested isolate is recorded in binary (0/1) code, generating a 'spoligotype' for each isolate. The spoligotype can be shortened into an octal code format. This method has several advantages for typing MTB: it is a simple procedure, robust and with high reproducibility, it can be applied directly on smear-positive sputum and a global database for comparison between laboratories is available (197). This technique is widely used in both evolutionary and epidemiological studies (198). Based on spoligotyping patterns, MTB can be divided into

its major families (199). However, spoligotyping is not discriminatory for strains of the East-Asian/Beijing lineage (lineage 2), and therefore is of limited use in Asia. The Beijing genotype lacks spacers 1-34 on spoligotyping. Spoligotyping is useful for discrimination between isolates of MTB with only few copies of *IS6110* (200), which are usually of lineage 1 (Indo-Oceanic) and are also common in South-East Asia. In addition, spoligotyping tends to homoplasmy (independent mutation events causing the same genotype) based on the evidence of phylogenetic unrelated isolates share the same spoligotype.

1.7.2.3 Mycobacterial Interspersed Repetitive Units - Variable Number Tandem

Repeats (MIRU-VNTR) typing

Mycobacterial Interspersed Repetitive Units (MIRU) is the term used for variable number tandem repeat (VNTR) typing in mycobacteria. MIRU are groups of repetitive units distributed throughout the mycobacterial genome, which were first described by Supply *et al.* They are direct tandem repeats and are oriented in one direction relative to the transcription of the adjacent genes. Based on the sequence homology of MTB sequences, it was estimated that there may be 40-50 such MIRUs per genome (201). Further work by Supply *et al.* identified a total of 41 MIRU loci when analysing the complete genome of the type strain H37Rv (Figure 1.6) (202). These tandem repeats varied between loci and strains and may be amplified with the use of specific PCR primers to each MIRU-flanking region. The number of repeats at each locus is calculated based on sizing of these PCR products, generating a numerical sequence: the MIRU genotype. Genotyping based on the Variable Number Tandem Repeats on MIRU, so called MIRU-VNTR typing, is a well-described method. Exploration of the MIRU loci with optimal discriminatory power has resulted in a recommendation of a set of 15 MIRU loci for molecular epidemiologic studies and 24 loci for molecular phylogenetic studies (203-207). MIRU-VNTR types align with other phylogenetic markers and assigns MTB to its major lineages described above (188). The discrepancies sometimes observed between

spoligotyping and MIRU based classification schemes underline that MIRU-based classification may group MTB isolates that are phylogenetically close but appear distant if only judged based on their spoligotyping patterns (208).

MIRU-VNTR typing is also an extensively characterised tool for transmission studies (209). The more MIRU loci are used, the higher the discriminatory power. The old 12 loci format is more discriminating for isolates with a low copy number of *IS6110* only. When more than 12 loci are used, or MIRU analysis is combined with spoligotyping, the discriminatory power is similar to that of *IS6110*-RFLP analysis (205). Better resolution and reduced clustering rate for MTB belonging to the Beijing lineage has been shown by using an additional four hypervariable MIRU-VNTR loci (210).

MIRU-VNTR typing has some advantages: it is a reproducible method, generates digital results that are readily exchangeable between laboratories and ensures the availability of global databases (211). The method can be simplified even further by the utilization of multiplex PCR and automated DNA sequencing technologies (212) although a manual protocol is more suitable for low workload laboratories in resource limited setting.

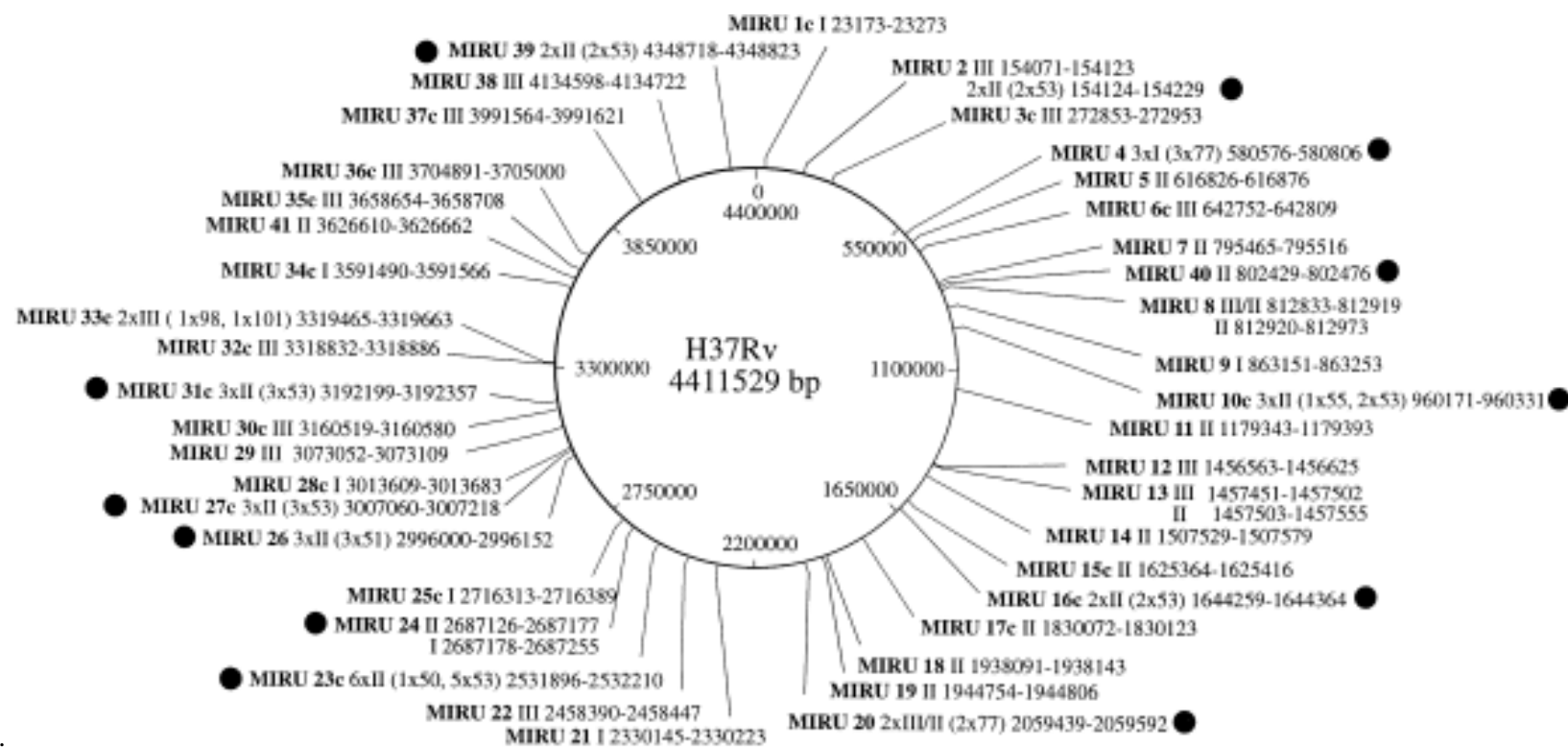


Figure 1.12 Location of MIRU loci in MTB H37Rv genome . Source from Supply et al. 2000 (202)

1.7.2.4 Whole genome sequencing

Whole-genome sequencing (WGS) technologies provide a complete genomic sequence including lineage characterisation and drug-resistant mutations (213-216). WGS is used in some developed countries to predict drug resistance in MDR-TB, but is not yet routinely applied to all TB cases due to high costs. It is likely to become more widely used as the costs decrease and simplicity of the technique increases. It is also the gold standard for outbreak investigation and tracking transmission (217). However, there are obstacles to implement these technologies in lower income countries, including the costs and specialised skills required for data interpretation. Several predictive software tools have been developed for MTB drug susceptibility prediction from WGS (218), but they are not yet field validated and our knowledge of resistance determinants is incomplete. Thus, older methods still have value, as they have a faster turnaround time and can handle a higher batch capacity at an affordable cost.

1.8 Outline of the thesis

Early and accurate diagnosis of TB will not only improve the outcome of treatment but also interrupt chains of transmission. During the past decades, improved diagnostic tools have shortened the time to detection and increased accuracy, but a true point of care test remains elusive (219). The characteristics of the ideal diagnostic test can be summarised by the ASSURED acronym (220): A-Affordable by those at risk of infection, S-Sensitive, S-Specific, U-User-friendly, R-Rapid, E-Equipment-free, D-Deliverable to those in need. Diagnostic tests for adult TB are still not sufficiently sensitive to rule out disease, and the challenges are amplified in paediatric TB. Children, particularly those under 5 years of age, do not produce sputum, the samples that are obtainable have very low bacillary loads, presenting symptoms are often non-specific and young children are unable to report symptoms.

Evaluation of novel diagnostic tests for TB is complicated by the lack of a gold standard for comparison due to the low sensitivity of culture in paediatric TB. However, it is vital that, as we develop new tests for the diagnosis of adult TB, we continue to evaluate them in children so this extremely vulnerable population can benefit from advances in TB research. In this thesis, therefore, we examine the application of available culture methods in TB detection in children living in endemic context.

The non commercial liquid culture assay, which is based on the microscopic observation of MTB growth using inverse microscopy (MODS) has been proven to have a similar performance as commercial culture assays regarding time to detection and accuracy in adults in various different settings (221). The data on the usefulness of this assay in diagnosing TB in children is limited therefore I aimed to evaluate the usefulness of this assay in a general paediatric hospital context in Vietnam, which is often the first point of contact with the health system for children with the general symptoms consistent with TB, such as fever and failure to thrive.

Confirmed diagnosis of tuberculosis in children remains challenging because of the difficulty of collected appropriate clinical specimens for testing. Therefore, specimens, which are easily collected, such as blood and urine, should be evaluated with assays for diagnosis of TB in children. In this thesis, therefore, I assessed the added value of mycobacterial blood culture to for diagnosis in children with TB suspicion.

Because of difficulty in specimen collection and limited diagnostic resources in endemic settings, confirmation of TB in children using culture is not often performed and even when MTB is successfully isolated, the drug susceptibility testing is not often performed due to additional expenses and lack of adequate infrastructure. Most endemic countries have limited

or no capacity of reliably performing TB culture and DST under appropriate biosafety conditions (222). In addition, childhood TB surveys are not a priority for most TB control programmes in endemic countries. Consequently, there is a shortage of data on drug resistance to anti-TB drugs in children, although this is a sentinel population for active transmission within a population. In this thesis, therefore, I also performed a molecular epidemiological analysis of drug susceptibility characteristics of TB in children in northern Vietnam.

In my thesis I aimed to answer the following questions:

- 1) What is the performance of MODS compared to conventional diagnostic methods in diagnosing TB in children?
- 2) Does adding mycobacterial blood culture increase the overall yield of TB diagnosis in children?
- 3) What is the prevalence of drug resistance in paediatric TB in Vietnam?
- 4) Is there an association between drug resistance and the lineage of MTB among children with TB in northern Vietnam?

The outline of the chapters is as follows:

- In Chapter 2 I describe the methodology used in the results chapters of the thesis.
- In Chapter 3 I describe a study to evaluate the accuracy of MODS assays compared to the conventional diagnostic methods in diagnosis of TB in children in the National Hospital of Paediatrics, the largest general paediatrics hospital in northern Vietnam, in Hanoi.
- In Chapter 4 I present the findings of a study to evaluate the additional value of mycobacterial blood culture in diagnosing TB in children in the same hospital and the paediatric department of the National Lung Hospital in Hanoi.

- In Chapter 5 I describe the first line TB drug susceptibility patterns of MTB from patients enrolled in the MODS study and explore the association between drug resistance and the genetic characteristics.
- In Chapter 6 I present an overview of the findings from my thesis, and I discuss the implications of the results and suggestions for further research to improve laboratory diagnosis of tuberculosis in children in resource-limited settings.

Chapter 2

Materials and Methods

2.1 Study settings

2.1.1 National Hospital of Paediatrics, Hanoi

The National Hospital of Paediatrics (NHP), established in 1981, is the largest hospital for general paediatric care in northern Vietnam with 1,100 beds. Annually, approximately 60,000 patients are admitted to NHP. The NHP microbiology department has a dedicated BSL2 laboratory for mycobacterial work where MODS was implemented in February 2009. Children with confirmed TB diagnosis are often referred to the National Lung Hospital (NLH) for free treatment and follow-up as per national guidelines except severe cases requiring paediatric intensive care unit (PICU) admission.

2.1.2 National Lung Hospital, Hanoi

The National Lung Hospital (NLH) is the nationally designated referral hospital for a range of pulmonary conditions, including adult and childhood TB. Children with suspected TB are referred to the NLH by several mechanisms: (1) self/family referral through the outpatient or emergency department, (2) from district or provincial hospitals throughout northern and central Vietnam, (3) and from the National Hospital of Paediatrics in Hanoi. Annually, there are approximately 800 children presenting at the paediatric department of NLH for TB treatment. The microbiology department of NLH functions as the national reference laboratory for TB diagnostics. The laboratory routinely performs tests for MTB detection and identification as well as drug susceptibility testing. MTB detection tests include smear staining, solid and liquid culture and molecular detection and typing assays. Drug resistance of MTB is determined by either phenotypic or molecular methods.

2.2 Sample size

2.2.1 Sample size calculation in MODS study

On a yearly basis, around 350-400 specimens for TB diagnostics are received by the NHP microbiology laboratory. Therefore, it is expected that 750 specimens from approximately 600 patients will be enrolled in this study. The estimated TB prevalence in our study population is estimated to be 20% based on the estimated TB prevalence in endemic countries (53), resulting in 120 positive cases in two years. At an estimated sensitivity of MODS to detect pulmonary TB of 90% (115), we will be able to assess to the sensitivity with the preciseness of $\pm 6.4\%$ (Appendix A)

2.2.2 Sample size calculation in mycobacterial blood culture.

The population of main interest were culture negative patients by all diagnostic tests of smear and gastric fluid (about 90% of the total study population). With the aim of this study to prove a “significant extra yield” by blood-culture in this population, the assumption was that the extra yield by blood culture would need to be at least 5% in the population of main interest. Based on this assumption, we need 795 patients to “prove” that the extra yield is $>3\%$ (i.e. to reject the null hypothesis that $\text{extra yield} \leq 3\%$), with a power of 80% (based on an exact binomial test with one-sided significance level 2.5%) (Appendix B)

2.3 Study timing and duration

The enrolment for the MODS study was conducted from February 2009 to December 2010. The enrolment of the mycobacterial blood culture study was carried out from June 2011 to December 2014. The enrolment of the drug resistance study was conducted from February 2009 to December 2013.

2.4 Scientific and ethical approval

All protocols, parental informed consent form (ICF) and case report form (CRF) were approved by the Institutional Review Board (IRB) at NHP and the University of Oxford Tropical Ethical Review Board (OxTREC), as applicable. The ICF form is shown in Appendix C .

2.5 Enrolment

2.5.1 Enrolment sites

MODS study

All patients with clinical symptoms suspicious of tuberculosis presenting to NHP were enrolled into the study of evaluation of MODS in diagnosis of TB (chapter 3).

Mycobacterial blood culture study

All patients with clinical symptoms suspected of tuberculosis presenting to NHP and the paediatric department of NLH were enrolled into the study of evaluation of mycobacterial blood culture study in diagnosis of TB (chapter 4)

Anti-TB drug resistance study

All patients with a new TB diagnosis at NHP and having a positive MODS culture with MTB were included in the study of characterising anti-TB drug resistance of MTB isolates (chapter 5).

2.5.2 Inclusion criteria

Patients suspected of having TB included in all studies met all of these following criteria: aged under 15 years, not or less than one week on TB treatment and having signs or symptoms of suspected TB. The specific requirement for patient enrolment of each study is explained in the method section of each chapter.

2.5.3 Exclusion criteria

TB suspected children whose conditions did not allow for collection of study specimens. For the study of evaluating mycobacterial blood culture, patients were not enrolled if written informed consent from patients or their parents/guardians was not obtained.

2.5.4 Data collection

Most data on socioeconomic and demographic features, TB history, TB contact history, HIV status and presenting clinical features were collected prospectively on a standard case report form (CRF). Exceptions are specifically mentioned in each chapter.

2.6 Definition and TB classification

2.6.1 TB case definition in MODS study

Patients were categorised into one of three groups: confirmed TB, probable TB or TB unlikely based on microbiological findings, intention to treat and outcome.

Tuberculosis was defined as "**confirmed TB**" if the patient had clinical signs and symptoms consistent with TB according to the guidance for national tuberculosis programmes on the management of tuberculosis in children by WHO (223) and either smear or LJ was positive in any sample, including samples which were collected before the enrolment started. These samples were not included in the sensitivity comparison but patients with prior samples positive in this illness episode by smear were classified in the "confirmed TB" group. A positive MODS culture was not considered as part of the definition of 'confirmed TB' because this was the test under evaluation.

The patient was defined as having "**probable TB**" on 'intention to treat' if the patient had clinical signs and symptoms consistent with TB but had no microbiological confirmation (yet), received no alternative diagnosis and initiated TB treatment and was transferred to a

NLH for treatment and follow-up. Patients who satisfied the first two characteristics of "probable TB" but self-discharged prior to treatment were also classified in this group if the clinician intended to treat for TB. It was impossible to either rule-out or confirm TB in this group due to the lack of microbiological confirmation.

Patients were defined as **"TB unlikely"** if they recovered without TB treatment, had TB treatment but deteriorated or received an alternative diagnosis and treatment. It was impossible to 'rule-out' TB in these patients completely because clinical deterioration on therapy may have been due to undetected drug-resistant TB. HIV screening is not a routine test for patients at NHP. HIV counseling and testing was offered if HIV was clinically suspected by the treating clinician, according to routine guidelines

2.6.2 TB case definition in blood mycobacterial study

Clinical case definition categories for TB in children were determined based on standardised case definitions recently published by Graham et al. as described in Figure 2.1 (75). TB signs and symptoms are defined as persistent unexplained fever, persistent cough, night sweats, weight loss, failure to thrive, reduced playfulness or lethargy, neonatal pneumonia, unexplained hepatosplenomegaly or sepsis like illness.

'Confirmed TB cases' were defined as children with microbiologically confirmed TB, defined as at least one positive smear or culture or WHO-endorsed nucleic acid amplification test (Xpert MTB/RIF) in any sample.

'Unconfirmed TB cases' were defined as children meeting at least 2 of the following criteria: defined signs or symptoms suggestive of TB; chest radiograph consistent with tuberculosis; close tuberculosis exposure or immunologic evidence of MTB infection, i.e positive tuberculin testing; positive response to tuberculosis treatment.

‘Unlikely TB cases’ were defined as children not meeting criteria for ‘confirmed TB cases’ and ‘Unconfirmed TB cases’.

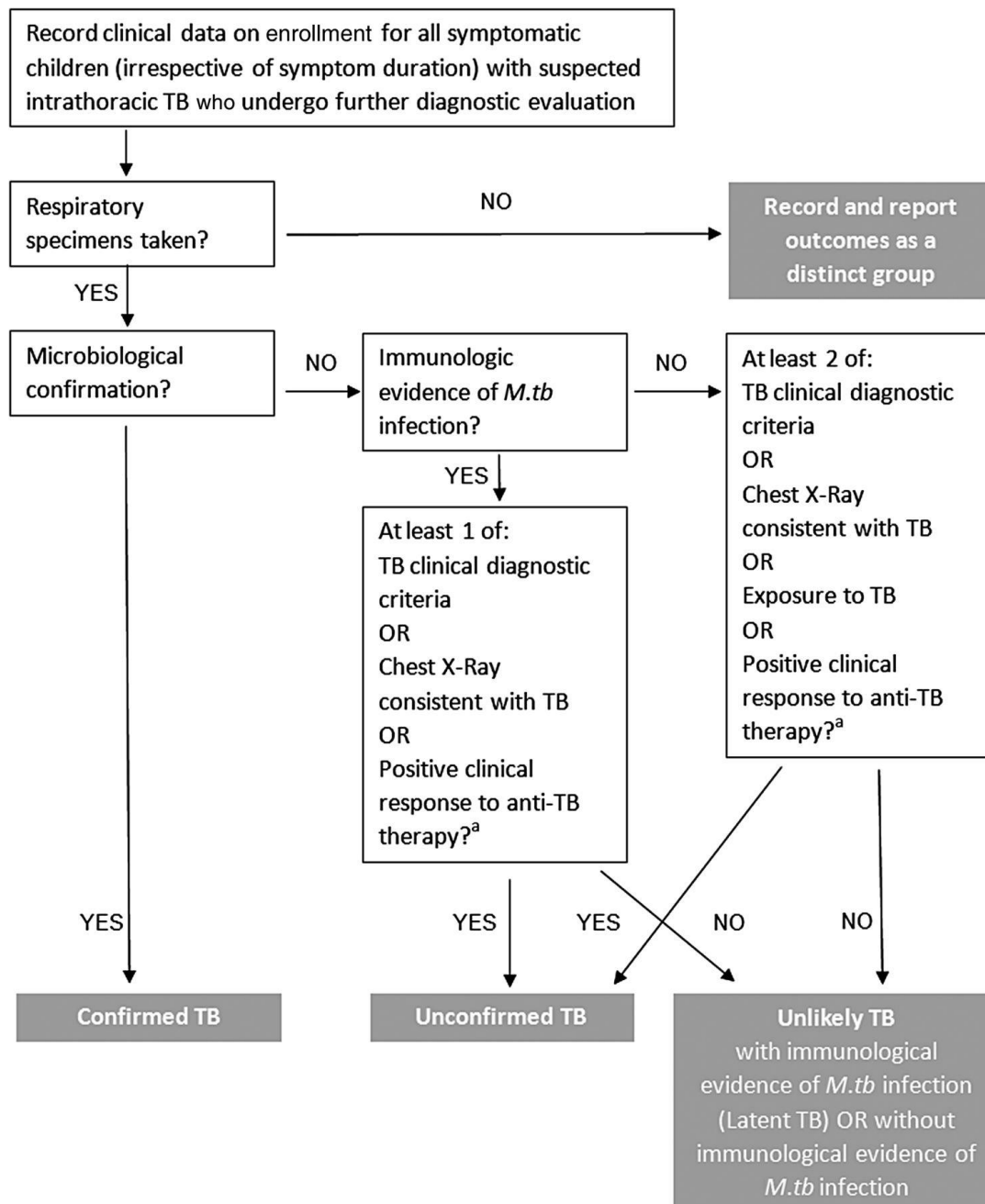


Figure 2.1 Algorithm for classification of case definitions in mycobacterial blood culture study.

Source from Graham *et al* 2015 (75)

2.7 Sample collection and transfer

2.7.1 Sample collection

The number of routine samples collected from patients was determined by the treating clinicians. In the MODS study, no additional samples were collected as part of this study. Blood samples were collected for the mycobacterial blood culture study. The collection procedure followed hospital routine practice. Gastric aspirates were not subjected to neutralization with bicarbonate on delivery because culture yield difference among neutralized and non-neutralized samples was not significant (224). Inadequate samples (insufficient volume, evidence of contamination or incorrect specimen) were rejected and resubmission was requested from the wards. All study specimens were collected prior starting anti TB therapy. Study clinicians were trained to collect and send specimens before starting TB treatment, or, as exception, no later than the first week of treatment. Date of start of treatment was, however, not recorded in the CRF.

2.7.2 Sample transfer

Blood specimen

Blood was collected after patient enrolment and sent to the microbiology laboratory. Samples from NLH were temporary incubated in a 37 °C incubator and then twice weekly transferred at room temperature to the NHP microbiology laboratory for 42 days incubation in automatic monitoring Bactec 9050 instrument (Becton Dickinson, USA).

Other specimens

Most samples were collected during working hours in weekdays and transferred to the microbiology laboratory for processing the same day. If the samples were collected outside of office hours, they were kept in refrigerator at the wards and were sent to the microbiology laboratory the next working day. Samples were kept cool on ice during transferring to NLH for LJ culture.

2.8 Laboratory methods

2.8.1 Sample preparation

All samples, except CSF, were decontaminated and homogenised with 0.5% N-acetyl-L-cysteine (NALC) (Sigma Aldrich, Wicklow, Ireland) - 4% NaOH (Sigma Aldrich, Wicklow, Ireland) solution. Briefly, the equal volume of NALC-NaOH was added to the sample tube. After mixing on the vortex, the sample tube was left standing for 20 minutes for liquefaction and decontamination. Then, sterile phosphate buffer solution (pH 6.8) (Sigma Aldrich, Wicklow, Ireland) was added up to 15 ml to neutralise the alkaline solution. Next step was to concentrate by centrifugation at 3000g for 20 minutes. CSF was not decontaminated before centrifugation. After discarding the supernatant, the pellet was resuspended in 0.5 ml PBS (Sigma Aldrich, Wicklow, Ireland). The diluted deposit was divided for smear preparation and culture isolation.

2.8.2 Ziehl-Neelsen (ZN) smear

Sample smear was prepared by dropping 2 drops of processed sample (approximately 0.1ml) onto the middle of a slide using a Pasteur pipette. Next, the smear was dried on a slide heater or at room temperature for 15 - 30 minutes, followed by heat-fixation using a Bunsen burner before staining by ZN method according to WHO standard protocol (225). Briefly, smears were flooded with 0.3% Carbon Fuchsin solution (supplied by the National Lung Hospital), followed by being heated on the flame of a Bunsen burner until steaming. Then, free stain was washed under running water. Next step was to decolourise the first dye with alcohol-acid solution (supplied by the National Lung Hospital). Lastly, the smear was stained with 0.3% Methylene blue solution (supplied by the National Lung Hospital). Under light microscopy, AFB appear as a pink-red slightly curved rod shape against a blue background.

2.8.3 Mycobacterial culture methods

2.8.3.1 Microscopic Observation Drug Susceptibility assay

Microscopic Observation Drug Susceptibility assay (MODS) uses a liquid medium containing MTB growth enhancing chemicals and antibiotic mixture for normal bacterial inhibition. The growth of MTB in MODS is observed under an inverted microscope and shows as strings or tangled cord.

MODS was performed as described in Park *et al.* (226) using the modification described by Caws *et al.*. Briefly, MODS media were prepared with 7.9 g Middlebrook 7H9 broth (Difco, Sparks, MD, USA), 4.13 ml glycerol (Sigma, USA) and 1.67 g bactocasitone (Difco, Sparks, MD, USA) in 880 ml sterile distilled water. The media were autoclaved and stored in 22 ml aliquots at 4 °C. Each new batch was tested for sterility by incubating one aliquot at 37 °C for 1 week. Before use, OADC (Oleic acid Albumin Dextrose Catalase) and PANTA (Polymycin Amphotericin B Nalidixic acid Trimethoprim Azlocillin) (Becton Dickinson, New Jersey, USA) were added into each tube to final concentrations of 5.5% and 0.22% to make working MODS media. Seven hundred and fifty microlitres of working MODS media was aliquoted to each well of a 48 well cell culture plate and 250 µl of processed sample was added. One positive control (*M.bovis* BCG) and one negative control well (sterile distilled water) were inoculated to each plate. Samples were inoculated into alternate wells to reduce potential for cross-contamination. 'Blank' wells contained MODS media only. To prevent cross-contamination from evaporation, plate seals (optical films, Biorad, California, USA) were used. The plate was incubated at 37 °C, and the result was recorded every alternate day after five days of inoculation for evidence of growth. Any cord formation including long cords or comma-shaped cords was recorded as positive and no cord was recorded as negative. The negative results were confirmed at day 30. Contamination was recorded if there was any non-mycobacterial growth (based on microscopy and colour) and/or turbidity in any negative control well.

2.8.3.2 Löwenstein-Jensen culture

Löwenstein-Jensen (LJ) is the most widely used medium for tuberculosis culture. It is an egg-based medium and contains glycerol/pyruvate which favours the growth of MTB complex/non-tuberculous mycobacteria (NTM), respectively and malachite green, which suppresses the growth of non-mycobacterial organisms.

The procedure of LJ culture was performed from the standard protocol by WHO (227). Two drops (0.1 ml) of processed specimen suspension were inoculated on each slope of LJ slant (supplied by the National Lung Hospital). The tube was then incubated at 37 °C in a slanted position for 24-48 hours and contamination was checked. After that, the tube was placed upright in racks for the remaining incubation time. The culture tube was examined weekly for bacterial growth. The positive growth of MTB was often detected within 3-5 weeks with the observation of rough, crumbly, waxy and non-pigmented colonies. However, if bacterial growth was observed within 5 days of incubation, rapidly growing mycobacteria were suspected. The culture is recorded as negative if no growth was observed after eight weeks. Quantitative results are recorded according to WHO guidelines. If NTM growth was suspected, a niacin test (see below) was performed. MTB was confirmed by a positive niacin test.

2.8.3.3 Mycobacterial Growth Indication Test

Mycobacterial Growth Indication Test (MGIT) from Becton Dickinson (New Jersey, USA) uses Middlebrook 7H9 broth for cultivation of mycobacteria. The bacterial growth results in the reduction of dissolved oxygen in the culture medium and subsequent release of fluorescent signals, which are otherwise quenched by free oxygen. The fluorescent signal is recorded automatically in the BACTEC™ MGIT™ 960 Mycobacterial Detection System.

The processed samples were subjected to MGIT culture, following the protocol of Becton Dickinson. In brief, 0.1ml PANTA, 0.5ml OADC and 0.5ml of each processed sample were added into a MGIT culture tube. The mixture was mixed by manual inversion and then incubated at 37 °C in the MGIT machine. Any positive MGIT culture was then subjected to ZN smear staining to determine presence of AFB. Positive AFB in culture broth confirmed MTB growth.

2.8.3.4 Mycobacterial blood culture

The recovery of mycobacteria in blood was performed with BACTEC™ Myco/F Lytic culture bottles (Becton Dickinson, Sparks, MD, USA) containing non-selective culture medium. The procedure followed the manufacturer's protocol. Briefly, venous blood (1-5mLs) was directly inoculated into a Bactec MycoF-lytic bottle. Then, the inoculated blood bottles were incubated in the Bactec 9050 instrument at 35 °C for 42 days by default. Bottles with a positive signal were checked for the presence of AFB by ZN smear staining. MTB identification was confirmed by GenoType MTBDRplus assay (Hain Lifescience, Nehren, Germany), which also provided the drug resistance to rifampicin and isoniazid. Samples with no growth signal by day 42 were considered negative.

2.8.4 Phenotypic drug susceptibility testing

Drug susceptibility testing (DST) was performed using the proportional method based on WHO guidelines (228). A pure culture of isolate in the active phase of growth (about 4 weeks) was used and previously characterised MTB were used as controls. In brief, bacterial suspensions were prepared by shaking several loops of colonies in a sterile flask containing glass beads. Next, the suspension was transferred to screw-capped tubes for adjusting the turbidity of the bacterial suspension to match the 1.0 McFarland standard with sterile distilled water, and then 10^{-2} and 10^{-4} dilutions were prepared. Finally, 200µl volume of suspension at the diluted concentrations of 10^{-2} and 10^{-4} were transferred to drug-free LJ slants and drug-

containing slants, respectively. The drug concentration was used as follows: rifampicin (40 µg/mL), isoniazid (0.2 µg/mL), ethambutol (2.0 µg/mL) and streptomycin (4.0 µg/mL). The inoculated slants were continuously incubated at 37 °C. The contamination was checked after 1 week of incubation and slants were read after 4 and 6 weeks of incubation for definitive interpretation. An isolate was considered to be susceptible if there were no colonies or the counted number of colonies was less than 1% of growth on the drug-containing medium compared with drug-free slant. An isolate was considered to be resistant if the number of colonies on the drug-containing medium exceeds that on the drug-free slant with the 1% inoculum. The DST was repeated when the colony count in the control tube was less than 200 or the percentage of resistant organisms appeared to approach 1%, by subjective assessment.

2.8.5 Niacin test

The niacin test detects the accumulation of niacin in culture media, which is used for the identification of MTB. Almost every strain of MTB excretes a large amount of niacin (nicotinic acid) into culture media. Niacin-negative MTB strains are very rare, and very few other mycobacterial species yield positive niacin tests.

The test was performed with paper strip BBL Taxo TB Niacin Tests Reagents (Becton Dickinson, New Jersey, USA) following the manufacture's instruction. In brief, 1ml of distilled water was added into a positive culture slant and allowed to cover the entire surface of the medium for 30 minutes. 0.5ml of the broth was then transferred to a sterile screw capped tube and a niacin paper was placed into the tube for 30 minutes. The result was interpreted based on colour change. If no colour was observed, a negative result was recorded. If the strip turned yellow, a positive result was recorded, confirming the presence of MTB.

2.8.6 Multiplex allele-specific PCR (MAS-PCR)

Genomic DNA of MTB isolates was extracted by heating method (see section 2.7.7.1). MAS-PCR to detect rifampicin resistance followed the procedure published by Tho *et al.* (229). Three separate PCR reactions were performed to amplify the RIF resistance determining region (RRDR) targeting hot spot codons 516, 526 and 531. Briefly, 5 µl DNA was added to the PCR mixture containing 0.75 U Taq DNA polymerase, 0.2 mM of each dNTP (Roche, Basel, Switzerland) and 125-400 nM of primers and 2 mM MgCl₂. The amplification programme was as follows: 95 °C for 1 min, followed by 35 cycles of 95 °C for 10 s 68 °C for 20 s, 72 °C for 20 s and a final step of 72 °C for 5 min. Negative control (water) and positive control were included in each PCR run. PCR products were analysed by electrophoresis on 1.5% at 120V for 40 min. The results were read with 2 bands for wild type isolates and only one band for mutant isolates.

MAS-PCR to detect isoniazid resistance was performed in a PCR reaction following the protocol developed by Tho *et al.* (230). The reaction containing three primer pairs: first one targeting *Hsp65*, which is specific for MTB and served as an internal control, the second one was used to detect the mutation C-15T in the *inhA* promoter region and the third one targets the *katG* region where the most common mutation (S315T) is located. The PCR reaction contained 1U Taq DNA polymerase, 0.2 mM each of dNTPs (Roche, Lewes, UK), 150-250 nM of primers, 2 mM MgCl₂ and 5 µl DNA templates. The amplification condition was as follows: 95 °C for 2 min, 10 cycles of (95 °C for 20s 68 °C for 60 s, 72 °C for 40 s) then 25 cycles of (95 °C for 20 s 68 °C for 20 s, 72 °C for 40 s) and a final step of 72 °C for 5 min. Negative control (water) and positive control (H37Rv) were included in each PCR reaction. PCR products were analysed by electrophoresis on 1.5% at 120V for 40 min. The PCR reactions yielded two bands for wild type isolates, a single band for *katG* 315 mutants and three bands for isolates mutated at *inhA* C-15T.

2.8.7 Mycobacterial Interspersed Repetitive Unit - Variable Number of Tandem Repeats (MIRU-VNTR) typing

MIRU-VNTR typing procedure followed the protocol described by Supply *et al.* (206). The simpler system using electrophoresis with agarose gels was used because it was reported to be as reproducible as the fluorescence-based DNA analyser based system at intra- and inter-laboratory levels, if appropriate quality control and quality assurance measurement were used (231, 232). The typing procedure consisted of three steps: DNA extraction, PCR amplification and electrophoresis.

2.8.7.1 DNA extraction

MTB isolates from MODS were subcultured in enrichment medium (7H9, Becton Dickinson, Sparks, MD, USA) supplemented with glycerol for one month. After that, one ml of culture medium was transferred into 1.5 ml tubes containing 500 µl of phosphate buffered saline (Sigma Aldrich, Wicklow, Ireland). The tubes were placed in a heating block at 100 °C for 15 min to heat lyse the cells. The tubes were vortexed at five minute intervals to assist in the heat lysis of the cells. Microtubes were centrifuged at 6000g for 2 min. The supernatant was transferred into a clean, labeled 1.5 ml tube. DNA template was stored at -20 °C.

2.8.7.2 PCR amplification

The 24 MIRU-VNTR loci consist of ten original MIRU-VNTR loci; six loci of exact tandem repeats (ETRs: ETR-A, -B, -C, -D, -E and -F), five Mtub loci (Mtub4, 21, 30, 38, and 39), and three Queen's University of Belfast (QUBs) loci (QUB-11b, -26, and 4156c). Each of 24 loci were amplified with specific primer sets described in

using the Hotstar Taq DNA polymerase kit (QIAGEN, Hilden, Germany) in separate PCR reactions. Reaction volumes of 20 µl containing 2 µl DNA template, 2.0 µl of 10X PCR buffer (supplied with kit), 0.2 µl of 20 pM primer set, 0.4 µl (100 µM) of each of the four deoxynucleoside triphosphates, dATP, dGTP, dCTP and dTTP, 0.4 - 4µl of 5 × Q-solution

(supplied with kit), 0.15 µl of Hotstar Taq (0.75 unit) and 8.8 -10.8 µl of pure H₂O. A DNA extract from MTB H37Rv was included in each set of reactions as a positive control and sterile distilled water as a negative control. The amplification protocol consisted of 15 min of denaturation at 95 °C, followed by 40 cycles comprising 1 min at 95 °C , 1 min and 30 s at 59 °C and 1 min at 72 °C and a final extension at 70 °C for 10 min.

2.8.7.3 Electrophoresis

The amplified products were electrophoresed on 1.5% (w/v) agarose gels (Sigma Aldrich, Wicklow, Ireland) with RedSafe™ dye (Sigma, 10mg/ml) with a final concentration of 0.5µg/ml in 1X Tris Borate-EDTA buffer (Sigma) at 110V for 30-60 minutes depending on the investigated loci. A 100 bp DNA ladder (Invitrogen, MA, USA) was included on the gel in every run. Product sizes were visualised and photographed using an ultraviolet (UV) transilluminator. Product sizes were established by Table 2.2comparing the bands with the DNA ladder bands. The number of repeats was estimated based on the Table 2.2 and Table 2.3

2.8.7.4 GenoType MTBDRplus assay

The GenoType MTBDR plus assay (Hain Lifescience, Nehren, Germany) is a strip based hybridization assay, or Line Probe Assay (LPA) which has the ability to identify rifampicin and isoniazid resistance. The identification of rifampicin resistance is enabled by detection of the most significant mutations of the *rpoB* gene (coding for the β-subunit of the RNA polymerase). For testing high-level isoniazid resistance (>2ug), the 315 position of *katG* (coding for the catalase peroxidase) is examined and for testing low-level isoniazid resistance, the promoter region of *inhA* (coding for the NADH enoyl ACP reductase) is analysed.

These tests were performed according to the manufacturer's protocol. Briefly, for amplification, 35 µl of a primer-nucleotide mixture (provided with the kit), amplification

buffer containing 2.5 mM MgCl₂, 1.25 U Hotstar *Taq* polymerase (QIAGEN, Hilden, Germany), and 5 µl of a preparation of chromosomal DNA in a final volume of 50 µl were used. The amplification protocol consisted of 15 min of denaturation at 95 °C, followed by 10 cycles comprising 30 s at 95 °C and 120 s at 58 °C; an additional 20 cycles comprising 25 s at 95 °C, 40 s at 53 °C, and 40 s at 70 °C; and a final extension at 70 °C for 8 min. Hybridisation and detection were performed in an semi automated washing and shaking device (Profiblot; Tekan, Maennedorf, Switzerland). The hybridisation procedure was performed at 45 °C for 0.5 h, followed by washing steps and the colourimetric detection of the hybridised amplicons. After a final wash, the strips were air dried and fixed on paper. The strips were pasted on the result-report sheet to analyse the results and stored protected from light.

2.9 MTB lineage classification

Isolates were assigned to the specific genotype lineages according to their 24 loci MIRU patterns by comparing with isolates in reference MIRU databases stored in the MIRU-VNTRPlus website <http://www.miru-vntrplus.org/MIRU/index.faces> (211). The MIRU-VNTRplus website is freely accessible and allows users to assign genotypes and compare these with global representative databases of different genetic markers including MIRU, spoligotype, large sequence polymorphism and single nucleotide polymorphism data. In this thesis, the definition of lineages was done by best-match analysis and tree-based identification using the categorical index (204). Isolates unclassified by MIRU-VNTR were assigned using the prediction algorithm on the TB lineage website http://tbinsight.cs.rpi.edu/run_tb_lineage.html (233). TB lineage uses a large collection of databases from most available datasets (US CDC, MIRU-VNTRplus, Brussels and SpolDB4) and can predict 7 seven major lineages using two different methods: rule-based systems (RULES) and conformal Bayesian network (CBN). In the RULES method, the isolates are classified based on the values of MIRU loci. In the CBN method, the major lineages are

predicted using a conformal Bayesian network. Some isolates that could not be assigned to a specific family by both databases were categorised as “unclassified”.

2.10 Statistics

Accuracy of diagnostic tests (sensitivity, specificity, positive and negative predictive values) was compared using McNemar’s test for patient analysis and a binary marginal generalised linear regression model (GLM) for sample analysis. The GLM is very flexible, allows for the inclusion of covariates and accounts for the fact that results of multiple samples from the same patient or test results of different tests on the same sample may be dependent.

Time to positive result of test was defined as the time period from sample processing to recorded final positive result. Time to positive result for different methods was compared using Wilcoxon signed rank test. Time-dependent sensitivity curves were estimated with the Kaplan-Meier method and samples without a positive test result were formally regarded as censored on day "infinity".

Proportion comparison of demographic and clinical features of patients between TB diagnoses was done with Chi-square test or Fisher's exact test for categorical data and the Kruskal-Wallis test for continuous data.

Association among factors was analysed using a logistic regression model. Factors with biological meaning and showing $P < 0.2$ in univariate analysis were included in the multivariate mode. All statistical analysis was performed using SPSS version 22 (IBM Inc, Armonk, New York, USA) and a P-value < 0.05 was considered as statistically significant.

Table 2.1 Primer sequences for the 24 MIRU-VNTR loci

PCR mixture set	Locus	Alias	Primer sequences (5' - 3')
Mix 1	580	MIRU 4; ETR D	GCGCGAGAGCCCGAACTGC
			GCGCAGCAGAAACGCCAGC
	2996	MIRU 26	TAGGTCTACCGTCGAAATCTGTGAC
			CATAGGCGACCAGGCGAATAG
	802	MIRU 40	GGGTTGCTGGATGACAACGTGT
Mix 2			GGGTGATCTCGGCGAAATCAGATA
	960	MIRU 10	GTTCTTGACCAACTGCAGTCGTCC
			GCCACCTTGGTGATCAGCTACCT
	1644	MIRU 16	TCGGTGATCGGGTCCAGTCCAAGTA
			CCCGTCGTGCAGCCCTGGTAC
Mix 3	3192	MIRU 31; ETR E	ACTGATTGGCTTCATACGGCTTTA
			GTGCCGACGTGGTCTTGAT
	424	Mtub04	CTTGGCCGGCATCAAGCGCATTATT
			GGCAGCAGAGCCCGGGATTCTTC
	577	ETR C	GTGAGTCGCTGCAGAACCTGCAG
Mix 4			GGCGTCTTGACCTCCACGAGTG
	2165	ETR A	AAATCGGTCCCATCACCTTCTTAT
			CGAAGCCTGGGGTGCCCGCGATTT
	2401	Mtub30	CTTGAAGCCCCGGTCTCATCTGT
			ACTTGAACCCCCACGCCCATTAGTA
Mix 5	3690	Mtub39	CGGTGGAGGCGATGAACGTCTTC
			TAGAGCGGCACGGGGGAAAGCTTAG
	4156	QUB-4156	CGTCCGAGCGACATCAC
			AGGATCGAGCGGTCCAG
	2163b	QUB-11B	CCGATGTAGCCCGTGAAGA
Mix 6			AGGGTCTGATTGGCTACTCA
	1955	Mtub21	AGACGTCAGATCCCAGTT
			ACCCGACAACAAGCCCA
	4052	QUB-26	GAGGTATCAACGGGCTTGT
			GAGCCAAATCAGGTCCGG
Mix 6	154	MIRU 2	TGGAATTGCAGCAATGGACCAACT
			TACTCGGACGCCGGCTCAAAAT
	2531	MIRU 23	CTGTCGATGGCCGCAACAAAACG
			AGCTCAACGGGTTGCCCCTTTGTGTC
	4348	MIRU 39	CGCATCGACAAACTGGAGCCAAAC
			CGGAAACGTCTACGCCCCACACAT

Mix 7	2059	MIRU 20	TCGGAGAGATGCCCTTCGAGTTAG
			GGAGACCGCGACCAGGTACTTGTA
	2687	MIRU 24	CGACCAAGATGTGCAGGAATACAT
			GGGCGAGTTGAGCTCACAGAA
	3007	MIRU 27; QUB-5	TCGAAAGCCTCTGCGTGCCAGTAA
			GCGATGTGAGCGTGCCACTCAA
Mix 8	2347	Mtub29	GCCAGCCGCCGTGCATAAACCT
			AGCCACCCGGTGTGCCTTGTATGAC
	2461	ETR B	GCGAACACCAGGACAGCATCATG
			GGCATGCCGGTGATCGAGTGG
	3171	Mtub34	GGTGCGCACCTGCTCCAGATAA
			GGCTCTCATTGCTGGAGGGTTGTAC

Final concentration of MgCl₂ (HotStart buffer already contains 1.5 mM MgCl₂) was 1.5 mM in mixes 3, 5, and 7; 2 mM in mixes 2 and 8; 2.5 mM in mix 6; and 3 mM in mixes 1 and 4

Table 2.2 Allele calling table of MIRU-VNTR

Allele	MIRU 02	Mtub04	ETRC	MIRU 04	MIRU 40	MIRU 10	MIRU 16	Mtub21	MIRU 20	QUB2163b	ETRA	Mtub29
0	402	537	171	175	354	482	565	116	437	67	195	335
1	455	588	208	252	408	537	618	149	514	136	270	392
2	508	639	266	329	462	590	671	206	591	205	345	449
3	561	690	324	406	516	643	724	263	668	274	420	506
4	614	741	382	483	570	696	777	320	745	343	495	563
5	667	792	440	560	624	749	830	377	822	412	570	620
6	720	843	498	637	678	802	883	434	899	481	645	677
7	773	894	556	714	732	855	936	491	976	550	720	734
8	826	945	614	791	786	908	989	548	1053	619	795	791
9	879	996	672	868	840	961	1042	605	1130	688	870	848
10	932	1047	730	945	894	1014	1095	662	1207	757	945	905
11	985	1098	788	1022	948	1067	1148	719	1284	826	1020	962
12	1038	1149	846	1099	1002	1120	1201	776	1361	895	1095	1019
13	1091	1200	904	1176	1056	1173	1254	833	1438	964	1170	1076
14	1144	1251	962	1253	1110	1226	1307	890	1515	1033	1245	1133
15	1197	1302	1020	1330	1164	1279	1360	947	1592	1102	1320	1190

Table 2.3 Allele calling table of MIRU-VNTR (continuous)

Allele	Mtub30	ETRB	MIRU 23	MIRU 24	MIRU 26	MIRU 27	Mtub34	MIRU 31	Mtub39	QUB26	QUB4156	MIRU 39
0	252	347	150	395	285	498	326	492	272	153	563	540
1	305	404	200	447	336	551	380	545	330	264	622	593
2	363	461	253	501	387	604	434	598	388	375	681	646
3	421	518	306	555	438	657	488	651	446	486	740	699
4	479	575	359	609	489	710	542	704	504	597	799	752
5	537	632	412	663	540	763	596	757	562	708	858	805
6	595	689	465	717	591	816	650	810	620	819	917	858
7	653	746	518	771	642	869	704	863	678	930	976	911
8	711	803	571	825	693	922	758	916	736	1041	1035	964
9	769	860	624	879	744	975	812	969	794	1152	1094	1017
10	827	917	677	933	795	1028	866	1022	852	1263	1153	1070
11	885	974	730	987	846	1081	920	1075	910	1374	1212	1123
12	943	1031	783	1041	897	1134	974	1128	968	1485	1271	1176
13	1001	1088	836	1095	948	1187	1028	1181	1026	1596	1330	1229
14	1059	1145	889	1149	999	1240	1082	1234	1084	1707	1389	1282
15	1117	1202	942	1203	1050	1293	1136	1287	1142	1818	1448	1335

Chapter 3

Diagnostic accuracy of Microscopic Observation Drug Susceptibility (MODS)

3.1 Introduction

In 2014 there were an estimated 1 million cases of paediatric tuberculosis (TB) and 130,000 associated deaths in the world (43, 234). The overall 13% global mortality rate is considerably higher than the 0.5% mortality rate in paediatric tuberculosis recorded in the United States and other developed nations (235, 236). While there are undoubtedly a number of contributing socio-economic factors, this gap highlights the need for accelerated diagnosis and initiation of appropriate chemotherapy in order to improve outcomes of paediatric TB cases within developing nations. Under-diagnoses of TB is a critical concern as children are failing to receive appropriate treatment (237).

Currently, diagnosis is complicated by three factors. First, high rates of extra-pulmonary disease contribute to non-specific clinical presentation of tuberculosis resulting in misdiagnosis (238-240). Second, adequate specimens for microbiological diagnosis are difficult to obtain because children often do not have a productive cough as presenting symptom and do not readily expectorate sputum. Finally, when samples are obtained, available tests are often insensitive, slow, or too expensive due to the paucibacillary nature of paediatric TB (241, 242). As a result, children who lack typical symptoms are seldom referred to TB hospitals where they can receive additional testing and appropriate treatment.

In Vietnam and other high TB burden countries, Ziehl-Neelsen (ZN) staining of acid-fast bacilli (AFB) is the primary method of diagnosis. While results are typically available within hours of sample collection, the test relies on the experience of the microscopist and is best suited for detecting higher concentrations of AFB. Direct smear is rarely positive in children (243). In addition to poor sensitivity, the test cannot distinguish between mycobacterium species and does not include susceptibility testing (239). In Vietnam, referral TB hospitals have access to more sensitive culture-based techniques. The most commonly used solid culture method is culture on Löwenstein-Jensen (LJ) media, which is more sensitive than ZN staining but requires long incubation times of up to 6 weeks (244-246). Commercial liquid culture systems, such as BACTEC MGIT 960 (Becton Dickinson, USA) and MB/BacT (Biomérieux, France), significantly reduce the time to detection and have higher sensitivities than LJ culture (9-22 days), and therefore should be used for the usually paucibacillary paediatric samples, but the high costs prevent routine use in resource-constrained settings (244, 247). The microscopic observation drug susceptibility (MODS) assay has been estimated to cost less than one dollar per test in 2007 and has been endorsed by WHO (117). MODS is a closed liquid culture system and has a low risk of contamination (248). The test was initially developed in Peru and was adopted nationally for TB diagnosis and rapid detection of MDR-TB. MODS culture has been evaluated in Ho Chi Minh City and Lima as a sensitive and rapid liquid culture technique for TB detection in paediatric specimens (249-251). While these MODS paediatric studies produced promising results, they were performed in populations in whom TB was highly suspected with a high pre-test probability of TB. These evaluations in referral populations may overestimate the clinical utility of the assay in a general population where the primary paediatric TB diagnosis is usually made. In addition, there have been limited reports of MODS for diagnosis of extra-pulmonary TB. To that end our study aimed to assess relative accuracy and time to positivity of MODS for diagnosis of both pulmonary and extra-pulmonary TB in children admitted to a large general paediatric

hospital in Vietnam. Direct susceptibility testing (inoculation of primary specimen into both drug-free and drug-containing wells) was not performed in this evaluation for paediatric samples, because of the paucibacillary nature of paediatric specimens and the high risk of many uninterpretable or false positive results due to bacterial inoculation into only one of the wells. This study therefore evaluated the culture technique of MODS for primary TB diagnosis only.

3.2 Methods

3.2.1 Study Design

The study design is summarised in Figure 3.1. The National Hospital of Paediatrics (NHP) implemented MODS to detect TB in children as of February 2009. Children with clinical suspicion of tuberculosis presenting at NHP, Vietnam were tested with ZN stain, MODS and LJ culture. In this study we evaluated MODS prospectively from February 2009 to December 2010.

Suspected TB cases were considered to have at least two of the following signs: unexplained fever for more than 1 week, unexplained cough for more than 1 week, radiographic findings suggestive of tuberculosis, failure to thrive or weight loss compared against the standard growth chart, malnutrition defined by WHO guidelines (25), enlarged non-tender lymph nodes or lymph node abscess, signs of meningitis with history of at least 1 week, HIV positive, contact with TB source within the preceding 2 years. Weight loss was defined as unexplained reduction in weight compared with the highest weight recorded in last 3 months. Failure to thrive or malnourishment was recorded if the weight for age or weight for height z score were equal or less than 2 in the normal growth chart. For HIV positive individuals, the definition included lack of response to nutritional rehabilitation or antiretroviral therapy. TB contact history was investigated by interviewing the patient or patient's parent. Any exposure

to a case of tuberculosis was considered as having TB history contact. The presence of a BCG scar on the arm of the patient was also checked by study clinicians. Data on demographic features, TB history, TB contact history, HIV status and presenting clinical feature were collected on a standard MODS test request form. HIV testing is not performed routinely at NHP and the decision to offer an HIV test is determined by the treating clinician. The treating clinicians collected samples according to routine protocols, depending on presenting symptoms.

Patients were categorised into one of three groups as previously described in chapter 2: confirmed TB, probable TB or TB unlikely based on microbiological findings, intention to treat and outcome. This study was designed before the recently published standardised case definition for research on childhood pulmonary TB (32).

3.2.2 Sample collection

For pulmonary TB diagnosis, two gastric fluid aspirates or two sputum samples were collected on successive mornings for microbiological testing at NHP. In case of suspected TB meningitis (TBM), cerebrospinal fluid (CSF) was obtained with a recommended minimum volume of one millilitre. For the other forms of TB suspected, other samples (bronchoalveolar lavage, pleural fluid, biopsy, or others as appropriate) were taken as clinically indicated at the discretion of the treating clinician. All specimen types referred for TB culture were assessed in this study. For each sample, one aliquot was reserved for smear and MODS culture at NHP, and another aliquot was transferred to the National Lung Hospital (NLH) for LJ culture. Morning specimens were processed for both culture methods on the same day of collection; specimens collected in different time in day were stored overnight at 4 °C and cultured on the following day.

3.2.3 Sample processing

All samples, except CSF, were homogenised and decontaminated with NaOH-NALC 2% following the protocol described in chapter 2. The supernatant was discarded and the pellet was re-suspended in 1ml PBS. This suspension was then aliquoted into two 1.5 ml vials, one for smear and one for MODS.

TB smear

Two drops of pellet from each sample were put onto a slide for homogenous smear preparation. The smears were then stained by Ziehl-Neelsen (ZN) method according to World Health Organization (WHO) standard protocol (252).

Löwenstein-Jensen culture

For culture at the National Lung Hospital, after decontamination, a drop of sediment was placed onto Löwenstein-Jensen (LJ) medium prepared according to international standards. LJ cultures were examined visually weekly for up to 8 weeks. Final identification of the isolates was performed according to standard recommendations.

MODS

MODS was performed as described by Caws *et al.* (117). More details are provided in chapter 2.

3.2.4 Contamination analysis

All MODS positive cultures were typed by 15-loci MIRU typing (206) to detect potential cross-contamination. The positive control culture inoculated to each MODS plate was an *M. bovis* BCG strain to enable easy detection of cross-contamination from the positive control. Furthermore, we left negative control (non-inoculated) wells in between inoculated wells to check for contamination.

3.2.5 Ethics

This study was approved by the Institutional Review Board of NHP. The need for informed consent was waived by NHP ethics committee as the study was considered to be an evaluation of medical services and did not alter routine patient care at NHP.

3.2.6 Statistical Methods

Accuracy measures of smear, LJ and MODS were calculated on clinical diagnosis (clinical gold standard including confirmed TB group and probable TB group) as the reference test. In addition, we analysed data on a 'per patient' and 'per sample' basis. In per patient analysis, the patient was regarded as positive if at least one sample yielded a positive test result. Exact binomial confidence intervals for accuracy measures (sensitivities, specificities, positive and negative prediction values) were calculated with the epiR package for R (253). Comparisons of accuracies between tests were done using an Exact McNemar's test. For the time to detection analysis, we performed a log rank test on the 'days to positive culture' data for samples that were both LJ and MODS positive (254).

Demographic and clinical features of patients between all TB groups (confirmed, probable and unlikely) were compared using a Chi-squared test for categorical data and Kruskal-Wallis test for continuous data. When comparing only 2 groups at a time, Fisher's exact test was used for categorical data, and a Wilcoxon rank sum test for continuous data.

All reported confidence intervals are two-sided 95% confidence intervals and P-values ≤ 0.05 were regarded as statistically significant. All analyses were done with R program (255) or SPSS program version 22 (IBM Inc, Armonk, New York, USA)

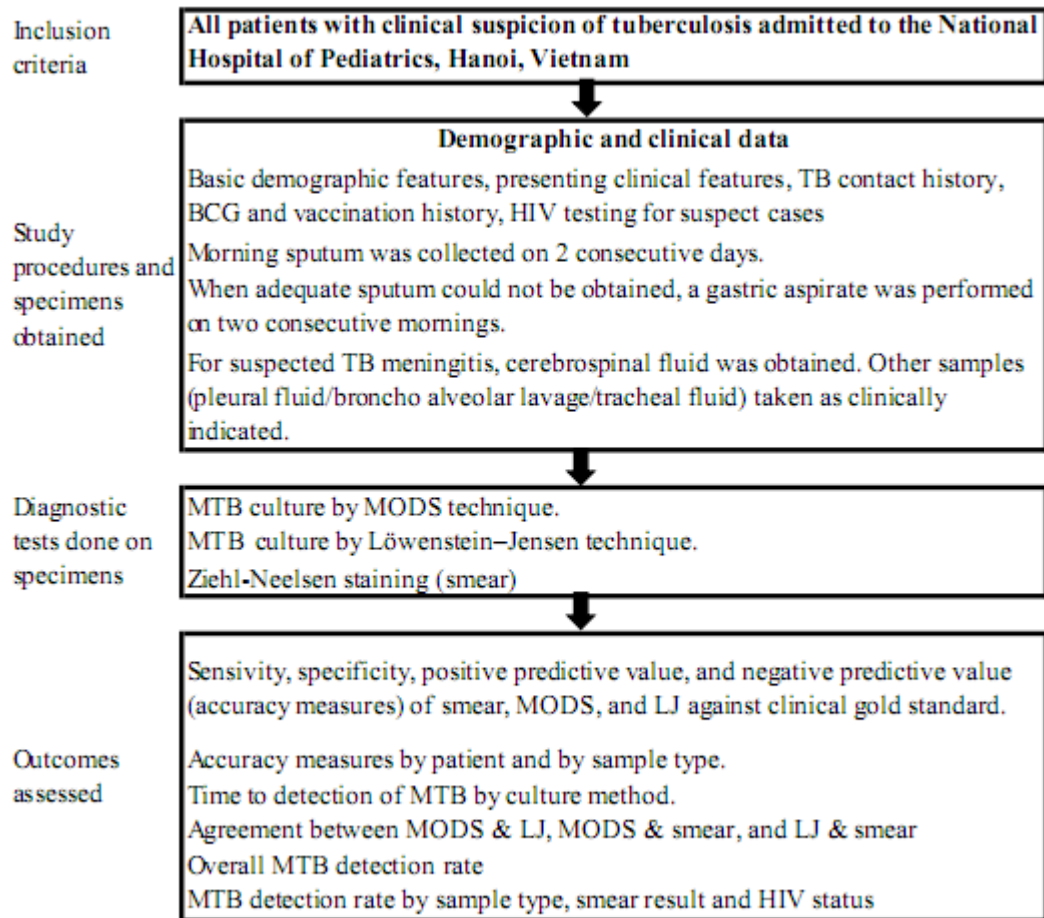


Figure 3.1 Study Design.

3.3 Results

From 1 February 2009 to 31 December 2010, 726 children suspected of having TB were evaluated in this study. Twenty-one patients were excluded from the analysis because there was insufficient specimen volume collected to be able to perform LJ culture. 1129 samples from 705 children remained: sputum (n = 59), gastric aspirate (n = 775), CSF (n = 148), pleural fluid (n = 33), bronchoalveolar lavage (n = 45), tracheal fluid (n = 41), other (n = 28). The other samples included nasal washes (n = 2), fine needle lymph node aspirates (n = 7), pericardial fluid (n = 1), nasopharyngeal aspirates (n = 8), ascites (n = 1) and synovial fluid (n = 1).

Forty-four patients (6.2%, n = 44/705) had microbiological confirmation by LJ culture ('confirmed TB'). Sixty-nine (9.8%, n = 69/705) patients were classified as 'TB probable' (see Figure 3.2). Among unlikely TB patients (n=592), 94.8% of patients (n=561) were diagnosed as having one of the following infectious disease syndromes: respiratory, non-TB (68.3%), central nervous system diseases (19.1%) or prolonged fever of unknown origin (7.3%).

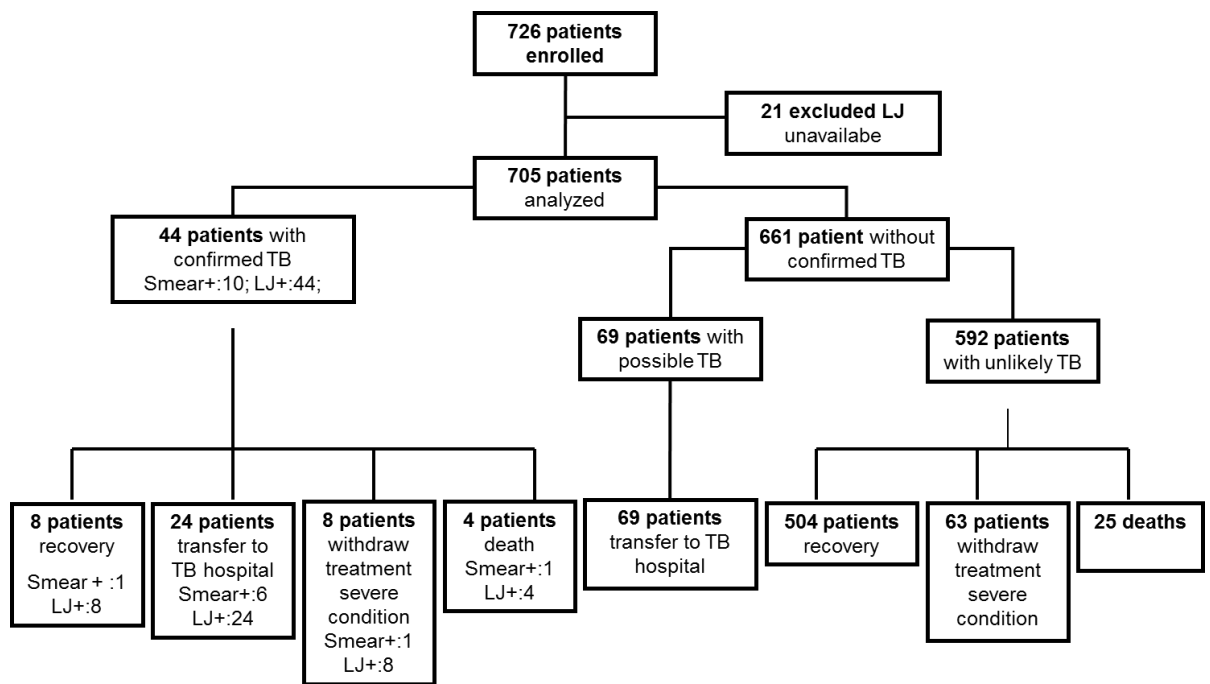


Figure 3.2 Patient recruitment and assignment to ‘confirmed TB’, ‘probable TB’, or ‘TB unlikely’ group.

3.3.1 Demographic characteristics

General demographic characteristics of the study population are presented in Table 3.1. In brief, the male to female ratio was 1.9:1 and the median age was 2 months (IQR = 1-48 months). The confirmed and probable TB cases were significantly older than the unlikely TB group ($P = 0.016$, $P = 0.001$, respectively). These findings could be explained by the possibility that older children produce better quality and more sputum in comparison to the young children. Only 55.7% had evidence of BCG vaccination. 9.2% ($n=65/344$ tested) of patients were HIV positive, and the confirmed and probable groups had significantly higher proportions of HIV positive children than the unlikely group: 18.2%, 15.9% and 7.7%, respectively ($P<0.05$). History taking showed that 6.5% of the children had a TB contact and 80.4% of those reported contacts were with a household family member. The confirmed and probable groups had a significantly higher proportion of reported TB contacts than the unlikely group: 15.9%, 17.4% and 4.6%, respectively ($P<0.05$).

3.3.2 Clinical Symptoms

Clinical records could not be obtained for 115 patients and therefore the data available only concerns 590 of the 705 study patients. Clinical symptoms of TB of this referral study population (Table 3.2) included cough (73.7%), fever (80.2%), weight loss (22.5%), lymphadenopathy (11.9%), malnutrition (28.5%), failure to thrive (27%), and chest X-ray consistent with TB (26.4%). The median history of illness (14 days, IQR = 8-20 days) did not vary significantly across groups. The confirmed TB group had a significantly higher proportion of patients with malnutrition (53.7%), weight loss (43.9%), failure to thrive (48.8%), and meningitis (51.2%) compared to the probable TB and unlikely TB groups (). The unlikely TB group had a significantly lower proportion of patients with chest X-ray suspected of TB than the confirmed TB and probable TB groups (21.8% versus 43.9% and 51.6%). Prevalence of lymphadenopathy was 22% amongst confirmed TB patients, 17.7%

amongst probable TB patients, and 10.3% amongst unlikely TB patients. The difference between confirmed and unlikely groups was statistically significant ($P = 0.035$).

Table 3.1 Demographic characteristics of patients

Category	Subcategory	Total Population N=705	Confirmed N=44	Probable N=69	Unlikely N=592	Comparison*
Age	Median (IQR) <i>in months</i>	2 (1-48)	23 (2-87)	36 (1-96)	2 (1-42)	P < 0.001 [P1=0.995; P2=0.001; P3=0.003]
	<5 years	545 (77.3)	29 (65.9)	41 (59.4)	475 (80.2)	P < 0.001 [P1=0.544; P2<0.001; P3=0.033]
Sex	Male	463 (65.7)	22 (50)	48 (69.6)	391 (66)	P = 0.073
BCG Vaccination	Yes	393 (55.7)	26 (59.1)	39 (56.5)	328 (55.4)	..
	No	44 (6.2)	5 (11.3)	5 (7.2)	34 (5.7)	P = 0.310
	Unknown	268 (38)	13 (29.5)	25 (36.2)	230 (38.9)	..
HIV Status	Positive	65 (9.2)	8 (18.2)	11 (15.9)	46 (7.7)	P = 0.009 [P1=0.8; P2=0.037; P3=0.025]
	Negative	279 (39.6)	17 (38.6)	36 (52.2)	226 (38.2)	..
	Unknown	361 (51.2)	19 (43.2)	22 (31.9)	320 (54.1)	..
TB Contact	Yes	46 (6.5)	7 (15.9)	12 (17.4)	27 (4.6)	P < 0.001 [P1=1; P2<0.001; P3=0.006]
	Yes & Contact was household family member	37 (80.4)	7 (100)	11 (91.7)	19 (70.1)	P = 0.111
	No	440 (62.4)	28 (63.6)	35 (50.7)	377 (63.7)	..
	Unknown	219 (31.1)	9 (20.5)	22 (31.9)	188 (31.8)	..

*Summary Measure for categorical data is n (%). * P is used to compare across all three groups (confirmed, probable, and unlikely). For continuous variables a Kruskal-Wallis rank sum test was used. When P < 0.05 Fisher's Exact test was used to compare confirmed with probable (P1), probable with unlikely (P2), and confirmed with unlikely (P3). For continuous variables a Wilcoxon rank sum test was used for P1, P2, and P3 calculations.*

Table 3.2 Clinical feature of 590 paediatric TB suspects

Characteristic	Total Population N=590	Confirmed N=41	Probable N=62	Unlikely N=487	Comparison*
Cough	435 (73.7)	32 (78.0)	48 (77.4)	355 (72.9)	P = 0.6048
Fever	473 (80.2)	37 (90.2)	52 (83.9)	384 (78.9)	P = 0.158
Malnutrition	168 (28.5)	22 (53.7)	15 (24.2)	131 (26.9)	P = 0.001 [P1 = 0.003; P2 = 0.761; P3 = 0.001]
Weight loss	133 (22.5)	18 (43.9)	10 (16.1)	105 (21.6)	P = 0.002 [P1 = 0.003; P2 = 0.41; P3 = 0.003]
Failure to Thrive	159 (27.0)	20 (48.8)	15 (24.2)	124 (25.5)	P = 0.005 [P1 = 0.012; P2 = 0.878; P3 = 0.003]
Lymphadenopathy	70 (11.9)	9 (22.0)	11 (17.7)	50 (10.3)	P = 0.027 [P1 = 0.619; P2 = 0.086; P3 = 0.035]
Meningitis	137 (23.2)	21 (51.2)	12 (19.3)	104 (21.4)	P < 0.001 [P1 = 0.001; P2 = 0.869; P3 < 0.001]
Chest X-ray suspected of TB	156 (26.4)	18 (43.9)	32 (51.6)	106 (21.8)	P < 0.001 [P1 = 0.546; P2 < 0.001; P3 = 0.003]
History of Illness: median (IQR) in days	14 (8 - 20)	14 (8 - 20)	15 (10 - 25)	14 (8 - 24)	P = 0.639

Summary Measure for categorical data is n (%)

** P is used to compare across all three groups (confirmed, probable, and unlikely). For continuous variables a Kruskal-Wallis rank sum test was used. When P < 0.05 Fisher's Exact test was used to compare confirmed with probable (P1), probable with unlikely (P2), and confirmed with unlikely (P3).*

3.3.3 Diagnostic Accuracy

The clinical reference standard was defined as patients who satisfied characteristics of either the “confirmed TB” group or the “probable TB” group. 113 patients (16%) were diagnosed with TB by clinical features and/or microbiological confirmation and 592 patients (84%) were classified as ‘TB unlikely’. See Table 3.3 for the diagnostic yield of three methods by disease spectrum in each patient group.

The sensitivity of MODS versus smear and LJ culture is summarised in Table 3.4. When analysed by patient or specimen, MODS was more sensitive than smear ($P < 0.001$ for both) and more sensitive than LJ culture ($P = 0.019$, $P = 0.015$). Specificity and positive predictive value (PPV) of MODS were 99.2% [95%CI: 98.0, 99.7] and 91.2% [95%CI: 80.7, 97.1] respectively. The negative predictive value (NPV) of smear, LJ culture, and MODS was 85.2% [95%CI: 82.3, 87.7], 90% [95%CI: 87, 91.8], and 90.6% [95%CI: 88.1, 92.7]. Further analysis on sensitivity of tests in different age groups is shown in Table 3.5. The MODS shows a significantly higher sensitivity than smear in all groups. When compared to LJ, the MODS was more sensitive but the difference did not reach statistical significance due to the small numbers of patients in each age group.

To investigate whether sample type had a strong impact on the sensitivity of the three methods, we investigated the number of each sample type collected from 113 clinically diagnosed TB patients and analysed the sensitivity of these methods in terms of sputum sample, gastric aspirate, and CSF. Our data in Table 3.4 show that MODS was more sensitive than smear in all sample types, and more sensitive than LJ culture only for gastric aspirates. Table 3.6 shows the diagnostic yield of three methods by sample type.

Of the 79 samples that were MODS positive only, five were collected from five patients in the “unlikely TB” group. The LJ subculture and molecular typing on these isolates confirm four

cases of MTB and one atypical mycobacterium. Inspection of inoculate position on culture plate and genotyping with MIRU-VNTR method ruled out the possibility of cross-contamination. Three of these five patients did not have enough remaining specimen for re-culture and the remaining two were discharged to die at home. If the additional four cases detected by MODS are considered to be true TB cases, the adjusted sensitivity “by patient analysis” of smear, LJ, and MODS are 8.8% [95%CI: 4.3, 15.7], 38.9% [95%CI: 29.9, 48.6] and 49.6% [95%CI: 40.0, 59.0], respectively.

Table 3.3 Diagnostic yield of smear, LJ and MODS by disease status among 705 children at NHP during 2009-2010

Group	Diagnosis	AFB (n, %)	LJ (n, %)	MODS (n, %)
Confirmed TB (n=44)	PTB (n=22)	6 (27.3)	22 (100)	21 (95.5)
	TBM (n=21)	4 (19.0)	21 (100)	20 (95.2)
	Others (n=1)	0 (0)	1 (100)	1 (100)
Probable TB (n=69)	PTB (n=52)	0 (0)	0 (0)	7 (13.5)
	TBM (n=11)	0 (0)	0 (0)	3 (27.3)
	Others (n=6)	0 (0)	0 (0)	0 (0)
Not TB (n=592)	Suspected PTB (n=407)	0 (0)	0 (0)	3 (0.7)
	Suspected TBM (n=101)	0 (0)	0 (0)	0 (0)
	Others (n=84)	0 (0)	0 (0)	1 (1.2)

n: number of MTB positive cases. PTB: pulmonary tuberculosis. TBM: tuberculous meningitis
 %: percent of positive cases in the diagnosis group

Table 3.4 Sensitivity of MODS, LJ culture, and smear against clinical gold standard

	Sensitivity n (%) [95% CI]			P-Value*	
	MODS	LJ	Smear	vs LJ	vs Smear
By patient (N = 113)	52 (46) [36.6, 55.6]	44 (38.9) [29.9, 48.6]	10 (8.8) [4.3, 15.7]	0.019	<0.001
By all samples (N = 195)	69 (35.3) [28.7, 42.5]	59 (30.3) [23.9, 37.2]	11 (5.6) [2.8, 9.8]	0.015	<0.001
By sample type:					
Sputum (N = 23)	8 (34.8) [16.4, 57.3]	7 (30.4) [13.2, 52.9]	1 (4.3) [0.1, 21.9]	0.500	0.008
Gastric Aspirate (N = 103)	28 (27.2) [19, 36.8]	20 (19.4) [12.3, 28.4]	3 (2.9) [0.6, 8.3]	0.004	<0.001
CSF (N = 33)	22 (66.6) [48.1, 82]	22 (66.6) [48.1, 82]	3 (9.1) [1.9, 24.3]	0.688	<0.001

The sensitivities of MODS, LJ culture, and smear were calculated against the clinical gold standard. Sensitivities were calculated by all samples (some patients provided more than one sample), by patient, and by sample type. Exact binomial 95% confidence intervals were calculated.

**Comparison of sensitivities using Exact McNemar's Test: MODS vs LJ and MODS vs Smear*

Table 3.5 Comparison of sensitivity of MODS, smear, LJ versus clinical standard in different age categories

By age (years).	Sensitivity n (%) [95% CI]			P-Value*	
	MODS	LJ	Smear	vs LJ	Vs Smear
0 to < 2	24 (48.0) [33.7, 62.6]	21 (42.0) [28.2, 56.8]	6 (12.0) [4.5, 24.3]	0.29	< 0.001
2 to < 5	9 (45.0) [23.1, 68.8]	8 (40.0) [19.1, 64.0]	0 (0)	0.25	0.001
5 to < 10	10 (41.7) [22.1, 63.4]	7 (29.1) [12.6, 51.1]	2 (8.3) [1.0, 27.0]	0.13	0.004
>= 10	9 (47.4) [24.5, 71.1]	8 (42.1) [20.3, 66.5]	2 (10.5) [1.3, 33.1]	0.5	0.008

The sensitivities of MODS, LJ culture, and smear were calculated against the clinical gold standard. Sensitivities were calculated by all samples (some patients provided more than one sample), by patient, and by sample type. Exact binomial 95% confidence intervals were calculated.

Table 3.6 TB diagnostic yield by specimen type in 1129 samples collected from 705 paediatric patients admitted to NHP.

Specimen type	Number of positive samples (n)		
	AFB	LJ	MODS
Gastric aspirate (n=775)	3	20	31
Tracheal aspirate (n=41)	0	2	2
Pleural fluid (n=33)	0	0	1
BAL (n=45)	3	6	5
Sputum (n=59)	1	7	8
CSF (n=148)	3	22	24
Nasal wash (n=1)	0	0	1
Lymph node aspirate (n=7)	1	1	1
Joint pus (n=1)	0	1	1
Others (19)	0	0	0
All (n=1129)	11	59	74

AFB: Acid Fast Bacilli Staining; LJ: Löwenstein-Jensen culture; MODS: Microscopic Observation Drug Susceptibility assay; BAL: bronchoalveolar lavage

3.3.4 Time to Detection

Time to detection was defined as the number of days from sample processing (day 1) to when results were available. Smear results were available on the same day as processing. Median time to detection using MODS was faster than LJ culture, 7 days (IQR: 6-11) vs. 35 days (IQR: 32-40). Six among seventy-four MODS positive samples (8%) were detected after three weeks. In the 55 samples that were positive by both MODS and LJ culture, MODS was faster in all 55 samples (100%) and the median time difference was 25 (20-29) days in favour of MODS (log rank $P < 0.001$) (see Figure 3.3).

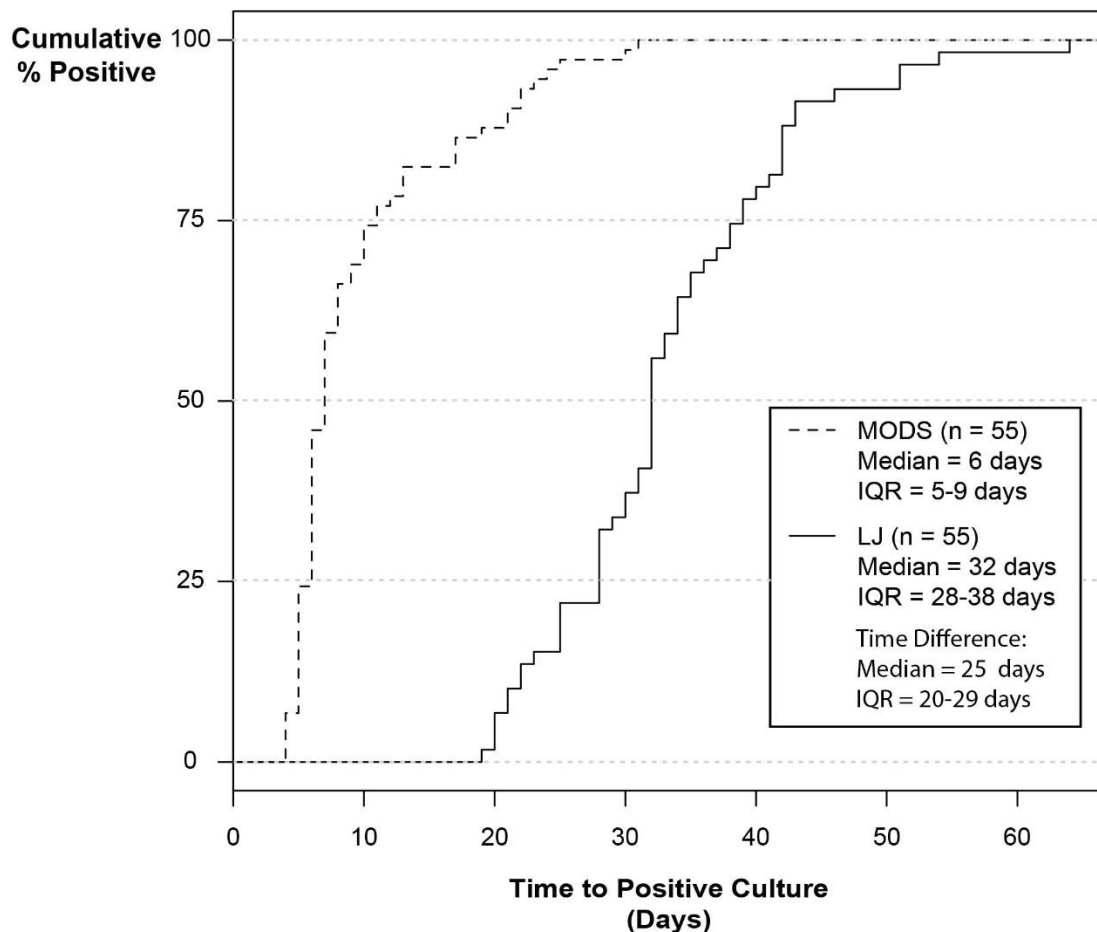


Figure 3.3 Paired Time to Detection by MODS and LJ Culture.

3.3.5 Contamination

The MODS contamination rate was low (26/1129, 2.3% of wells), and most contamination was due to fungi in samples from HIV patients. Cross-contamination was not observed during this study period.

3.4 Discussion

This study presents a direct comparison between MODS culture, LJ culture, and smear, across multiple specimen types, for diagnosis of paediatric pulmonary and extra-pulmonary tuberculosis in a large Vietnamese general paediatric hospital. MODS proved to be a relatively sensitive and rapid technique for the diagnosis of paediatric TB. MODS was significantly more sensitive than both smear (46% vs. 8.8%) and LJ (46% vs. 38.9%), and significantly faster than LJ with a median time difference of 25 days in favour of MODS. The sensitivity of MODS (46%) was slightly higher than the sensitivity reported in a paediatric study (39.7%) performed in HCMC, Vietnam (250). For smear negative patients in our study, the sensitivity of LJ and MODS was 33% [95%CI: 24.1, 43] and 41.7% [95%CI: 32.1, 51.9], respectively. Most of the improved yield from MODS, as compared to LJ, in our study can be attributed to an improved yield from gastric aspirates where MODS sensitivity was 27.2% and LJ sensitivity was 19.4% (Exact McNemar Test $P = 0.004$).

The time to detection was also significantly shorter for MODS than LJ. The recently proposed MODS standard operation procedure states that if no growth is observed after day 21, the result is recorded as negative (59). However, in this study 6 specimens were positive after day 21. Therefore, based on the finding in this study, the authors recommend that MODS should be incubated for at least one month.

In comparison with the HCMC study that was performed at a TB referral hospital, our study had a higher MTB recovery rate from CSF samples ($33/148 = 22.3\%$ vs. $4/30 = 13.3\%$), and therefore a higher detection rate of paediatric TB meningitis. Nine of the 33 patients with positive CSF samples also had respiratory specimens positive for MTB. It is possible that the increased recovery rate from CSF samples in NHP can be attributed to a difference in disease spectrum and empirical anti-tuberculosis treatment of patients with TBM prior to referral.

Furthermore, the participants in our study were much younger than those in the HCMC study. The mean (IQR) age of participants in the HCMC study was 9 years (3-13 years) whereas the mean (IQR) age of TB confirmed patients in our study was 24 months (10-87 months) (239). Our study results therefore show that MODS has diagnostic utility in very young children (241).

This study has several limitations. Firstly, several patients were lost to follow-up at the TB hospital and their classification in the “probable TB” group may have been a misclassification, which would overestimate the specificity of the MODS assay. Patients may have been misclassified as probable TB when in fact they did not have TB due to a number of reasons. Once a patient had begun TB treatment this misclassification was difficult to detect. Treatment response is often not a reliable indicator of TB disease as patients may have undiagnosed drug resistance, deteriorate despite adequate therapy, or conversely, have a non-TB bacterial infection, which responds to antituberculous drugs or a self-resolving viral illness and therefore improve while on TB treatment without having TB. The impossibility of definitively categorising cases is a recognised limitation in all studies assessing TB diagnostics in children (31). The use of ‘intention to treat’ rather than a strictly defined case definition for probable TB was chosen to represent the spectrum of disease in a high-burden clinical setting. A strictly defined case definition will likely overestimate the sensitivity of a

diagnostic test for TB (due to some true TB cases being misclassified as ‘not TB’), while a broad case definition (as used here) will usually overestimate the specificity due to some non-TB cases being classified as TB. Although the MODS assay can be used to test for drug susceptibility, DST was not performed by MODS in this study due to the paucibacillary nature of paediatric samples. Further study is needed to confirm if MODS direct DST is applicable for paediatric specimens.

The GeneXpert system (Cepheid, Belgium) is being implemented with rapid scale-up in developing nations as part of the WHO monitored global rollout plan (256). The GeneXpert MTB/RIF assay is more sensitive than smear microscopy, performed in under two hours, safe for technicians, and accurate for detecting rifampicin resistance (257). A cost-effectiveness analysis of TB diagnosis demonstrated that GeneXpert MTB/RIF use for initial diagnosis of suspected TB cases would substantially decrease TB associated mortality and morbidity at a reasonable cost, according to conventional cost-effectiveness benchmarks (258). However, the accompanying sensitivity analysis shows that higher usage of culture methods would result in a less favourable cost-effectiveness ratio for GeneXpert MTB/RIF. GeneXpert MTB/RIF should not be considered a replacement for culture methods, especially considering the need to test for resistance beyond rifampicin (259). Moreover, in regards to paediatric TB, various studies showed the suboptimal sensitivity of GeneXpert MTB/RIF as compared to the culture. In recent meta-analysis, the pooled sensitivities of GeneXpert MTB/RIF for pulmonary tuberculosis detection in children were 62% (95% CI: 51-73) using sputum samples (induced or expectorated) and 66% (51-81) using gastric lavage in compared with culture (127). A study of 452 suspected cases performed across two hospitals in Cape Town, South Africa found that 25% of children with culture confirmed tuberculosis were negative by the GeneXpert MTB/RIF assay (260). Similar findings also were reported in a study of 150 suspected children in HCMC, in which more than 30% of MGIT culture positive cases failed

to be detected by GeneXpert MTB/RIF (98). Although the implementation of the GeneXpert MTB/RIF will increase case detection and considerably shorten time to treatment for those children who are diagnosed, given the high rate of false negatives and a negotiated cartridge price of \$10 USD/test, GeneXpert MTB/RIF alone will not be the solution for paediatric TB diagnosis in resource constrained settings (261).

There was one misclassification of NTM as MTB in this study (1/69 positive MODS samples) due to cording observed in the MODS well. This issue has been addressed by a revision to the MODS standard operating procedure that includes additional wells containing p-nitrobenzoic acid (PNB) to distinguish MTB from NTM growth (59). The agreement of MTB species identification between MODS and LJ increased from 94.8% to 100% when adding PNB (111).

In conclusion, MODS is a rapid low-cost diagnostic tool for TB diagnosis in the paediatric population. General paediatric hospitals in TB endemic countries can improve their TB diagnosis by implementing MODS in case other liquid culture systems are absent. Access to the MODS assay increases TB case-finding and early diagnosis, which can shorten the time to appropriate therapy.

Chapter 4

Mycobacterial blood culture for diagnosis of TB in children

4.1 Introduction

Diagnosis of childhood TB is difficult due to the unspecific clinical signs and symptoms and difficulty in microbiological confirmation (234). Most guidelines recommend isolation of MTB from any sample and a positive culture is considered the gold standard for TB diagnosis in children (262-264). Young children typically are unable to expectorate sputum; therefore gastric aspiration is used instead. However, this procedure is invasive and may require hospital admission. Alternative specimen types, such as induced sputum or string test are unpleasant and not well tolerated by young children (84). Therefore, use of a more easily accessible specimen, such as blood or urine should be evaluated for its applicability in clinical diagnostics in paediatric TB.

The isolation of MTB from blood has been attempted in TB suspects with a high risk of mycobacteraemia, such as HIV infected (265, 266), extrapulmonary TB suspects or children (267). The yield of blood cultures in detecting MTB varied between 2% and 64% depending on the study population (268). A study of HIV infected individuals from South-East Asia found a 5% incremental yield of blood culture for TB from adults (265, 269-271). It is known that young children also are at higher risk of TB bacteraemia due to an immature immune system (234, 272) but there is limited evidence of the utility of blood culture for childhood TB diagnostics. Therefore, we undertook a prospective study to investigate the additional yield of mycobacterial blood culture for TB diagnosis in children at two tertiary hospitals in northern Vietnam. Mycobacterial blood culture was compared with other liquid culture methods using the standardised case definition.

4.2 Methods

Patient enrolment

Any child (< 15 years of age) presenting at NHP or the paediatric ward of NLH with suspected TB was eligible to join the study if they met all the following requirements: (1) having at least two inclusion criteria (see below); (2) not receiving TB treatment or having TB treatment less than one week; (3) and written informed consent from their parents/guardians. The inclusion criteria are: unexplained fever (>2 weeks), unexplained cough (>2 weeks), chest X-ray suspicious for TB, evidence of malnutrition or failure to thrive/loss of weight, lymph adenopathy, suspected meningitis (> 1 week), HIV infection or contact with TB cases. Inclusion criteria were broad in order to recruit all children with any suspicion of TB in the differential diagnosis.

One 1-5 ml blood sample and other routine diagnostic samples were collected for the study. The number and type of routine specimens were judged by the treating clinicians. All specimens were collected before starting anti-TB therapy.

Case definition

Clinical case definition categories for TB in children were determined retrospectively and taken from the standardised case definition published by Graham *et al.* (75). Three categories were defined including: confirmed TB cases, unconfirmed TB cases and unlikely TB. The definition of each TB category is described in chapter 2, section 2.5.2.

Laboratory testing

All patients had blood collected for mycobacterial culture (chapter 2, section 2.7.3). All samples, except CSF, were decontaminated using the NALC-NaOH protocol (chapter 2, section 2.7.2). Sample pellets were then divided for smear microscopy and MODS or MGIT culture. Smear staining was performed following the WHO standard protocol (252) (chapter

2, section 2.7.2). All test results were informed to treating clinicians for diagnosis and following up.

Ethics

Eligible children were invited to participate in the study through their parents who gave written informed consent following consultation. The protocol, patient information sheet (PIS), informed consent forms (ICF) and case report forms (CRF) were approved by the NHP Institutional Review Board (IRB) and the Oxford Tropical Ethics Committee (OxTREC number 13-10).

Statistical analysis

We compared the demographic and clinical characteristics of patients between diagnosed categories of TB (confirmed TB cases, unconfirmed TB cases and unlikely TB cases) using Fisher's exact test for categorical variables and Kruskal-Wallis test for continuous variables. The simple proportion comparison was used to assess the incremental yield of mycobacterial blood culture. To determine risk factors for MTB bacteraemia, logistic regression was included in analysis. All analyses were done using SPSS version 21 (IBM Inc, Armonk, New York, USA) . Two sided P-values < 0.05 were regarded as statistically significant.

4.3 Results

4.3.1 Study population

From July 2011 to December 2014, a total of 554 suspected cases of paediatric TB were enrolled into the study. A recruitment flowchart is shown in Figure 4.1. Using the standardised case definition (chapter 2, section 2.5.2), 92 patients (16.6 %, n = 92/55) were classified as 'confirmed TB', 345 patients (62.3%, n = 345/554) were 'unconfirmed TB' cases, 73 patients (12.3% n = 73/554) were 'unlikely TB' cases and 44 patients, having no routine samples, were classified as a separate group, which was excluded from analysis.

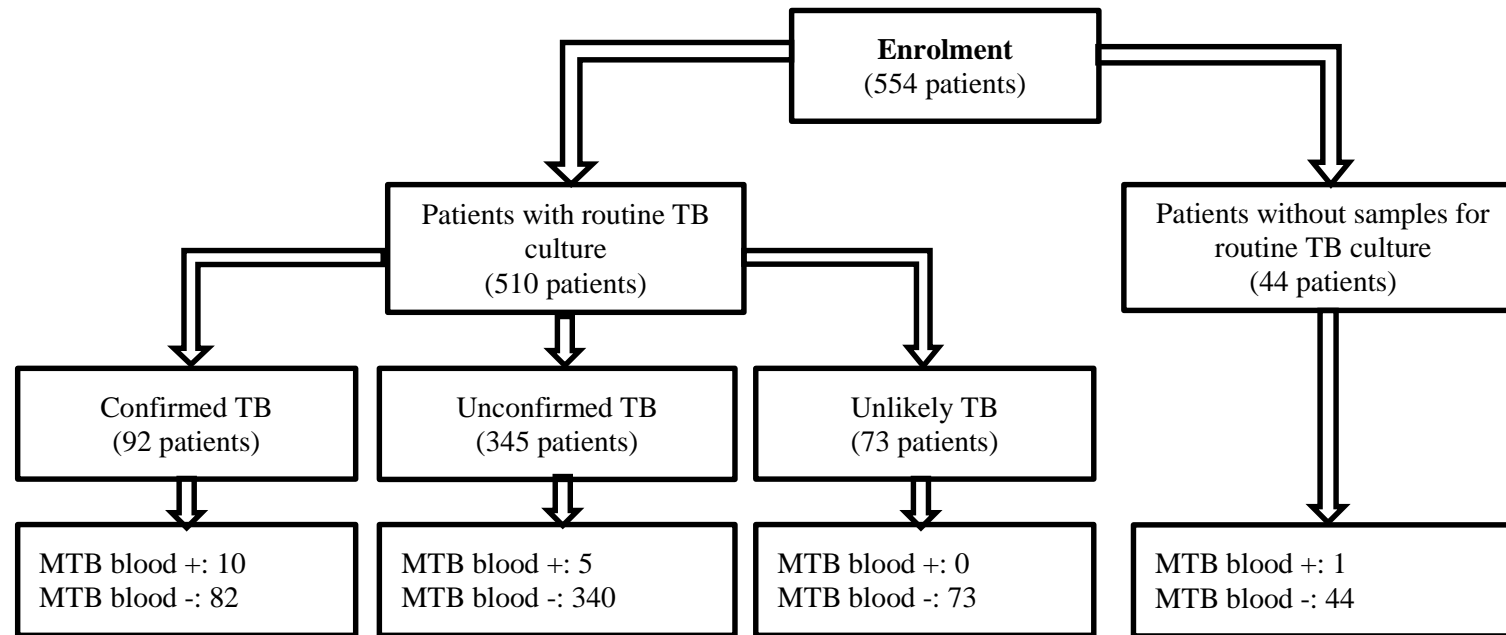


Figure 4.1 Flowchart of diagnosis for patients included in the study showing final TB diagnosis and mycobacterial blood culture

The treating clinician determined the number and type of routine samples collected. In total, 1028 samples were collected from 510 patients including: sputum (n = 81), gastric aspirates (n = 831), and others (n = 116). There were 125 patients from whom one sample was taken, 263 patients had 2 samples taken, 111 patients 3 samples and 11 patients had 4 samples taken. General demographic characteristics of the study population are shown in Table 4.1. Overall, the median age of children in the study population was 29.9 months (IQR: 10.9-85.2). Evidence of BCG vaccination was recorded in 92.2% of patients (n = 470/510) (scar or parent report). Neonatal BCG vaccination is compulsory under the Expanded Vaccination Program (EVP) of Vietnam. Over one-fourth (25.3%, n = 129/510) of the study population had a TB contact within the previous one year according to parent interviews and of those contacts 12% (n = 15/129) were a household member.

The clinical presentation of patients reported in this study population included fever (79.4%), persistent cough (82.4%), weight loss (44.2%), failure to thrive (54.9%), lymphadenopathy (11.5%) and chest X-ray consistent with TB (61.7%) (Table 4.2). Over 70% (n = 384/510) of the children had a TST done; 127/384 (33.1%) of these were positive. The median history of illness for all children in the study was 30 days [IQR 6.45-90].

Table 4.1 Demographic characteristics of 510 patients in different TB categories.

Characteristics	Total	Patients in different TB categories (N, %)			p-value
		Confirmed	Unconfirmed		
		TB N=92	TB N=345	Unlikely TB N=73	
Male	323 (63.1)	49 (53.3)	224 (64.9)	50 (68.5)	0.12
Age (months)					
Median [IQR]	29.9 [10.9-85.2]	39 [7.5-150.8]	29.6 [11.5-81.5]	23.5 [12.5-65.4]	0.47
BCG vaccination					
Yes	470 (92.2)	84 (91.3)	316 (91.6)	70 (95.9)	0.908
No	38 (7.5)	8 (8.7)	27 (7.8)	3 (4.1)	
Unknown	2 (0.4)	0	2 (0.6)	0	
TB contact					
Any contact	129 (25.3)	30 (32.6)	96 (27.8)	3 (4.1)	0.036
Family member	15 (2.9)	3 (3.3)	11 (3.2)	1 (1.4)	
No contact	146 (28.6)	19 (20.7)	116 (33.6)	11 (15.1)	
Unknown	220 (43.1)	40 (43.5)	122 (35.4)	58 (79.5)	
HIV infection					
# of HIV tests done	223	47	157	19	0.716
# positive HIV tests	13 (5.8)	2 (4.3)	11 (7.0)	0 (0)	

** p- value for comparison of all 3 groups. Total of 510 patients were classified into three TB categories: confirmed TB (92 patients); unconfirmed TB (345 patients), unlikely TB (73 patients)*

Table 4.2 Clinical characteristics of patients by final clinical classification

Characteristics	Total: N (%)	Patients in different TB categories: N (%)			p-value ^{\$}
		Confirmed TB N=92	Unconfirmed TB N=345	Unlikely TB N=73	
Fever (> 38oC)	404 (79.4)	79 (82.6)	276 (80.0)	49 (68.1)	0.017
Prolong fever > 2wks	229 (56.7)	60 (76.0)	209 (75.7)	30 (61.2)	0.132
Cough	420 (82.4)	76 (82.6)	285 (82.6)	59 (80.8)	0.934
Persistent cough > 2wks	362 (86.2)	57 (75.0)	253 (88.8)	52 (88.1)	0.117
Weight loss	211 (44.2)	50 (58.1)	140 (43.6)	21 (30.0)	0.002
Failure Thrive	261 (54.9)	49 (57.6)	168 (52.3)	44 (63.8)	0.192
CXR consistent to TB	309 (61.7)	77 (86.5)	229 (66.8)	3 (4.3)	< 0.001
Lymphadenopathy	54 (11.5)	16 (19.5)	26 (8.2)	12 (17.4)	0.004
Positive tuberculin skin test*	127 (33.1)	48 (63.2)	79 (33.6)	0 (0)	< 0.001

*) % positive tuberculin skin test (TST) was calculated from 384 among 510 patients who had Mantoux tests done. ^{\$})p-value for comparison of all 3 groups. Total of 510 patients were classified into three TB categories: confirmed TB (92 patients); unconfirmed TB (345 patients), unlikely TB (73 patients). CXR: chest X-ray.

4.3.2 Overall yield of mycobacterial blood culture

Of 554 blood culture specimens, a positive signal was recorded in 32 samples from which MTB was isolated in 16 (2.9%) (Table 4.3). Of the remaining positive blood cultures, *Talaromyces marneffe* (formerly: *Penicillium marneffe*) was found in 2 (6%). The median time to detection of positive MTB growth in 16 blood culture samples was 25 days (IQR: 13.5-29).

Table 4.3 Results of mycobacterial blood cultures for diagnosis of tuberculosis in children

Test results	Test done	%
Negative	522	94.2
Positive	32	5.8
<i>Mycobacterium tuberculosis</i>	16	2.9
<i>Staphylococcus epidermidis</i> *	9	1.6
<i>Talaromyces marneffei</i>	2	0.4
<i>Burkholderia cepacia</i> *	2	0.4
Unclassified	3	0.5

**Staphylococcus epidermidis* and *Burkholderia cepacia* were considered to be contaminants.

4.3.3 Comparison of mycobacterial blood culture to other cultures

Of the 16 patients with MTB bacteraemia, there were 15 patients that also had a routine sample collected for comparison (Table 4.4). Only two among these 15 cases were AFB smear positive. As compared to routine culture, positive MTB growth in blood was found in 5 cases whose routine culture was negative (Figure 1). Mycobacterial blood culture increased the total number of TB cases having bacteriological confirmation from 92 to 97 cases (5% added to the overall confirmation yield). The results of GenoType MTBDRplus assay showed that all 6 additional cases were sensitive to RIF and INH. Among 28 disseminated cases determined by clinical diagnosis, the overall yield of MTB positive blood culture was lower than that of routine liquid culture (6/28 , 21.4% vs. 21/28, 75%) although mycobacterial blood culture detected one additional case for whom routine culture was negative. When analysing 15 mycobacteraemia cases having submitted routine samples, the median time to detection was 26 days (IQR: 13.0- 31.0) for mycobacterial blood culture and 14 days (IQR: 8.8 – 16.0) for routine culture.

Table 4.4 Diagnostic yield of 16 cases having MTB isolation from blood

Patient ID	Routine specimen tested	AFB smear results	Routine culture results	TAT of positive routine culture (days)
20-018	NA	NA	NA	NA
20-029	Gastric aspirate 1 st	Neg	Neg	NA
20-043	CSF	Neg	Pos	23
	Gastric aspirate 1 st	Neg	Neg	NA
20-065	Gastric aspirate 2 nd	Neg	Pos	9
	Gastric aspirate 1 st	Neg	Pos	7
	CSF	Neg	Neg	NA
	Gastric aspirate 3 rd	Neg	Neg	NA
20-097	Gastric aspirate 1 st	Neg	Pos	14
	CSF	Neg	Pos	11
20-106	Gastric aspirate 1 st	Pos	Pos	9
22-017	Gastric aspirate 1 st	Neg	Pos	8
	Gastric aspirate 2 nd	Neg	Neg	NA
22-046	Sputum	Neg	Pos	5
22-053	Gastric aspirate 1 st	Neg	Neg	NA
	Gastric aspirate 2 nd	Neg	Neg	NA
22-066	Gastric aspirate 2 nd	Neg	Pos	16
	Gastric aspirate 1 st	Neg	Pos	10
22-072	Gastric aspirate 1 st	Neg	Neg	NA
	Gastric aspirate 2 nd	Neg	Neg	NA
22-084	Gastric aspirate 2 nd	Neg	Pos	16
	Gastric aspirate 1 st	Neg	Pos	15
22-101	Gastric aspirate 1 st	Neg	Neg	NA
22-133	Gastric aspirate 1 st	Neg	Neg	NA
22-242	Gastric aspirate 1 st	Neg	Pos	16
	Gastric aspirate 2 nd	Neg	Pos	15
	CSF	Neg	Neg	NA
22-327	Gastric aspirate 2 nd	Pos	Pos	17
	Gastric aspirate 1 st	Neg	Pos	13

NA: not available; Neg: negative result; Pos: positive result; CSF: cerebrospinal fluid; TAT: turnaround time

4.3.4 Characteristics of patients having MTB bacteraemia

The demographic and clinical characteristics of patients having MTB isolated from blood are summarised in Table 4.5. Among 16 cases having MTB bacteraemia, 10 cases presented with disseminated TB (3 having tuberculous meningitis and 7 having miliary TB). Two patients with positive mycobacterial blood culture were found to be HIV infected. In the bivariate analysis, only HIV infection showed a significant association with mycobacteraemia ($p = 0.046$). Outcome is considered as unfavourable if the patients died at the hospital or the patients withdrew from treatment to go home because of the severity. Unfavourable outcome was more frequently found in TB patients having MTB isolated from blood (26.7% versus 5.5%, $p = 0.01$) (Table 4.6).

One among 16 cases with MTB bacteraemia had no routine culture taken and blood was the only sample to detect MTB. That patient was HIV infected and presented with persistent cough, prolonged fever and a CXR consistent with TB. The patient's condition progressed quickly to severe on admission and no further samples were collected before the patient withdrew from treatment to go home.

Among 5 cases having mycobacterial blood culture as the sole source of MTB, there were 2 cases who received TB treatment with a final clinical diagnosis of miliary TB. The remaining 3 cases did not receive TB treatment at the time of discharge before the positive MTB growth in the blood sample was released.

Table 4.5 Characteristics of 16 patients with mycobacteraemia.

Patient ID	Sex	Age (months)	HIV status	Cough	Fever	Malnourished	Weight loss	Failure to Thrive	Lymph adenopathy	Meningitis	TB suspected CXR	Site of evidence of TB	TB Treatment	Outcome	MTB identified by routine culture
20-043	F	7	ND	-	+	-	-	+	-	+	+	TBM	+	Withdrew	+
20-065	F	2	ND	+	+	-	-	-	-	+	+	Miliary TB	+	Withdrew	+
20-097	F	144	ND	-	+	-	-	-	-	+	NA	TBM	-	Survived	+
20-106	F	15	-	+	+	-	-	-	+	+	+	Miliary TB	+	Withdrew	+
22-017	F	9	-	+	+	+	NA	+	-	+	+	PTB	-	Withdrew	+
22-046	M	84	+	+	+	+	+	+	-	+	+	PTB	+	Survived	+
22-066	M	2	-	+	+	-	+	+	-	+	+	Miliary TB	+	Survived	+
22-084	F	5	-	+	+	+	-	+	NA	NA	+	PTB	+	Survived	+
22-242	M	13	-	+	+	+	+	+	NA	+	+	Miliary TB	+	Survived	+
22-327	F	102	ND	+	+	-	+	+	+	+	+	Miliary TB	-	Survived	+
20-018	F	77	+	+	+	-	-	-	-	-	+	Bacteraemia/HIV	-	Withdrew	-
20-029	M	1	+	-	-	-	-	-	-	+	NA	Meningitis	-	Survived	-
22-053	M	4	ND	+	+	-	-	+	-	+	+	PTB	+	Survived	-
22-072	M	82	-	+	+	+	+	+	-	+	+	PTB	-	Survived	-
22-101	F	48	-	+	+	+	+	+	-	+	+	Miliary TB	+	Survived	-
22-133	M	13	ND	+	+	NA	NA	NA	NA	NA	-	Pneumonia	-	Survived	-

* Cases having negative routine culture

ND: not done, NA: not available, M: male, F: female, PTB: pulmonary tuberculosis, TB: tuberculosis, (+/-): present/absent,

Table 4.6 Characteristics of 437 TB diagnosed patients (including confirmed TB and unconfirmed TB)

Characteristics	Total	Patients with TB diagnosis (N, %)		P-value *
		Mycobacteraemia present (n=15)	Mycobacteraemia absent (n=422)	
Male	273 (62.5)	7 (46.7)	266 (63.0)	0.28
Age (months) Median [IQR]	55.9 [10-92]	13.0 [4-82]	31.5 [10-93]	0.05
BCG vaccination				
Yes	400 (91.5)	12 (80.0)	388 (91.9)	0.21
No	35 (8.0)	3 (20)	32 (7.6)	
Unknown	2 (0.5)	0	2 (0.5)	
TB contact				
Any contact	126 (28.8)	7 (46.7)	119 (28.2)	0.31
Family member	14 (3.2)	1 (6.7)	13 (3.1)	
No contact	135 (30.9)	4 (26.7)	131 (31.)	
Unknown contact	162 (37.1)	3 (20.0)	159 (37.7)	
HIV infection (204 patients tested)	13 (6.4)	2 (22.2)	11 (5.6)	0.046
Unfavourable outcome*	27 (6.2)	4 (26.7)	23 (5.5)	0.01

* Unfavourable outcome includes deaths at the hospital and withdraw treatment.

4.4 Discussion

This is the first prospective study to evaluate the added value of mycobacterial blood culture in diagnosis of TB in children with suspicion of TB. The study population represents the diversity of TB manifestations in children from two large referral hospitals in an endemic TB setting, in which approximately half of children have a clinical presentation of extrapulmonary TB. The study compared the detection rate of MTB from blood samples

versus that from routine samples. Therefore, this study allows us to determine the true added yield of mycobacterial blood culture for TB diagnosis in children.

The culture confirmation rate in children with a final TB diagnosis in this study (28.4%, 98/345) was low, similar to that in most other studies performed in the paediatric setting (93, 273). In a recent study by Giang *et al.* in a TB referral hospital in southern Vietnam, there were approximately 25% of cases with a positive MGIT culture (98).

The findings in this study show that including blood culture to routine MTB diagnostics may marginally increase the overall yield of TB detection. Mycobacterial blood culture confirmed six TB cases for whom culture of other specimens was negative (5 cases) or unavailable (1 case). However, the added diagnostic yield from blood culture was low as compared to the substantial additional cost with a number needed to test of approximately 70 per additional case detected with a unit cost for one bottle of Myco F/lytic of 7,50 USD, excluding the extra cost of identification reagents, consumables, maintenance and labour. Therefore, the costs for the Myco F/Lytic bottles alone are 525 USD per additional case detected.

Blood specimens could be considered an attractive sample for diagnosis of TB in young children because of the higher frequency of haematogenous spread and disseminated disease. However, unlike our expectation of high frequency for mycobacteraemia in disseminated cases, only 21.4% cases were detected using blood culture as compared to 75% from respiratory samples using routine culture methods. An explanation for the low detection rate of MTB in blood may be that the mycobacteraemia is transient and occurs before presentation at the hospital and blood collection, or that the mycobacteraemia only occurs in a minority of children.

In previous studies in adults (265, 274), blood culture positivity for mycobacteria was more frequently found in HIV infected patients and this difference was also observed in our study population (22.2% in HIV positive (2/15) vs. 5.6% in HIV negative (11/422), $p = 0.046$). However, not all children were tested for HIV infection and the number of the children with confirmed HIV infection in this study was small ($n=13/223$ tests), therefore, we can not comment on the true increased yield of mycobacterial blood culture for HIV infected children.

The time to positivity of mycobacterial blood culture in children with suspected TB in this study was long (median TAT: 25 days), which is similar to TAT found in studies in adults (265, 275, 276). For example, in a study by Diep *et al.* among patients with prolonged fever in Vietnam, median TAT of mycobacterial blood culture was found as 24.5 (275). The slow turnaround time of mycobacterial blood culture seems to be unsuitable for early detection of TB in children and results would not significantly change the clinical decision to treat or not to treat at an early stage (33). Therefore, methods to shorten the time to detection of mycobacteria in blood are needed if blood culture is to have clinical utility. Recently it was found that detection of MTB in blood using GeneXpert MTB/RIF could produce results within a day (277). The sensitivity of GeneXpert MTB/RIF was found to be similar to that of mycobacterial blood culture in a study of 104 HIV infected adults (33) but the high volume of blood used here (20 ml) would not be feasible in children. Another disadvantage of blood culture for MTB detection is the requirement of additional confirmation steps for MTB, which increases the laboratory workload and limits the utility of this test in most hospitals in countries with high burden of TB. GeneXpert MTB/RIF testing does not require a second confirmation step as the amplification target is specific for MTBC.

This study has some limitations. The study used the standardised case definition of intrathoracic TB to define all TB forms, which may result in the misclassification of some cases. However, it is always difficult to encompass the entire spectrum of possible presentations of paediatric TB. A too broad standardised research case definition will include ‘false positive’ TB cases, and underestimate the specificity of diagnostic tests, while a too narrow case definition will exclude true TB cases, and consequently overestimate test sensitivity. The case definition has recently been revised and it is likely it will continue to be refined as validation studies are conducted. Further case definitions for EPTB in children are needed to standardise research methods but will be very hard to establish. The standardised research case definition tool is not intended to be used as a diagnostic tool to guide research decisions, but rather to standardise clinical research on paediatric TB, including clinical trials and diagnostic evaluations. This will also facilitate comparison between sites and data synthesis into meta-analysis from multiple small studies. The decision to collect blood culture was dependent upon the attending clinician and therefore some cases would have been missed. Data were not available for eligible cases during the study period that were not enrolled. Despite these limitations, our study demonstrates the utility of mycobacterial blood culture to TB detection in children.

In conclusion, mycobacterial blood culture contributes little to the overall routine diagnostic yield for diagnosis in children and cannot be recommended for use in resource-constrained settings.

Chapter 5

***Mycobacterium tuberculosis* genotype and drug resistance in paediatric tuberculosis in northern Vietnam**

5.1 Introduction

Tuberculosis (TB) in children reflects recent transmission and is a useful indicator of ongoing transmission in the community, including transmission of drug resistance (23, 278). Although surveillance data of childhood TB are of importance to measure the effectiveness of the local tuberculosis control programmes, it is rarely done in low and middle income countries (LMIC) with a high TB burden (185). The main reasons for this are the challenging nature of TB diagnostics in children (279) and the absence of systematic efforts to record data on paediatric TB.

Vietnam ranks 12th among 22 countries with a high burden of TB in the world (43). Burden is defined based on the absolute number of TB cases, not (relative) incidence. Children are usually excluded in national tuberculosis surveys (183). In instances that children are included in the survey, the number of children contributes only a small proportion to the analyses (0.2%, 3/1312) (179). As TB culture is usually not done, there are limited data on drug susceptibility in children in LMICs, including Vietnam.

It has been shown that certain genotypes of MTB are associated with drug resistance in some geographical locations, but data are often conflicting and the underlying mechanisms are not clearly understood. It is possible that discrepancies in drug resistance associations with lineages are due to differences in the circulating ‘background’ isolates compared, different

patterns of drug pressure in different populations, or simply that reported associations are due to chance. Global studies will help to elucidate the true nature of these associations. Some *in vitro* evidence appears to support a higher rate of drug resistance acquisition among the Beijing genotype isolates and a reduced fitness cost for this genotype of the resistance-conferring mutations once acquired (150, 151, 163, 164, 187). These data lend support to the epidemiological observations of an association. For example, the worldwide prevalent Beijing genotype harbours higher frequencies of particular drug resistance-conferring mutations and was shown to be more frequently associated with drug resistance in some countries like Russia (280), China (281) and India (282). Epidemiological studies in Vietnamese adults showed that there is an association between drug resistance and the Beijing genotype in Vietnam (283-286). In a study among adults in three rural districts in the south of Vietnam, the researchers found that the Beijing genotype was associated with any drug resistance (OR 3.7, $P < 0.001$) and multidrug resistance (OR 6.8, $P < 0.001$) among new patients (284). The Beijing genotype was also reported to be strongly linked to fluoroquinolone resistance (OR 11.0, $P = 0.001$) in adult patients with pulmonary TB presenting at a referral TB hospital in Ho Chi Minh city, Vietnam (285). However, it is unknown whether there is an association between drug resistance and genotype of MTB among children. This would be an important observation because MTB isolates from children are representative of active transmission in the community, whereas adult TB cases are a mixture of recently acquired and reactivated TB. In this study, we aimed to characterise the drug susceptibility to first line anti TB drugs and the associations between drug resistance and genotypes of MTB from children admitted to the National Hospital of Paediatrics, Hanoi, and Vietnam during 2009-2013. The findings of this study will help to better understand characteristics of the current circulating MTB isolates in the community.

5.2 Methods

Isolate collection

MTB isolated by MODS from children presenting at NHP during 2009-2013 were used in this study. These isolates were collected from participants in the studies in Chapter 3 and 4. Once a specimen was found to be positive for MTB, laboratory details were recorded regarding the date of sampling, and specimen site. Clinical case notes and laboratory data were reviewed and demographic and clinical data extracted. The isolate was tested phenotypically by the indirect 1% proportion method for susceptibility to isoniazid, streptomycin, rifampicin and ethambutol (chapter 2, section 2.7.4). Multiplex allele specific PCR (MAS-PCR) to detect rifampicin (229) and isoniazid resistance (230) were performed on the phenotypically INH resistant isolates (Chapter 2, section 2.7.6). The 24-loci Mycobacterial interspersed repetitive units–variable number tandem repeats typing (MIRU-VNTR) typing procedure was subsequently performed, following the protocol described by Supply *et al.* (Chapter 2, section 2.7.7) (206).

Lineage identification

Isolates were assigned to specific genotype lineages according to their MIRU patterns by comparing with the online reference database MIRUplus and the TB lineage website (chapter 2, section 2.8). Some isolates, which could not be assigned to a specific family, were categorised as “unclassified”.

Statistics

The relative proportion of different genotype families is reported, comparing drug-susceptible to drug-resistant isolates. Statistical analysis was performed using SPSS program version 22 (IBM Inc, Armonk, New York, USA). A P-value of < 0.05 was considered as statistically significant (two-sided). The Chi-square test or Fisher’s exact test was used to compare the proportions among groups of patients. To compare the age of patients infected with different

MTB lineages, the Mann-Whitney U test (pairwise comparisons) was used for continuous nonparametric data. Logistic regression was used to determine independent risk factors for infection with each lineage.

5.3 Results

5.3.1 Description of isolates

MTB isolates were collected from 125 consecutive previously untreated children with positive MODS at NHP over a period of 5 years (from 2009 to 2013). The demographic characteristics of patients are shown in Table 5.1. The median age of the population was 20.4 months (IQR: 7.6-91.3) with 65.6% (82/125) aged under 5 years. Approximately half of cases presented with extrapulmonary TB (61/125; 48.2%) of which tuberculous meningitis was the most common manifestation (49/61; 80.4%). HIV co-infection was documented in 19 patients (15.2%). The distribution of residential areas of the 125 patients included in this analysis is illustrated in Figure 5.1. Hanoi had the highest number of cases (39/125, 31.2%), reflecting the location of the hospitals, Nghe An was following (10/125, 8%), and the remaining cases were resident throughout northern Vietnam.

Table 5.1 Demographic and clinical characteristics of patients (n=125)

Characteristics	N	%
Male	68	54.4
Age months (median, IQR)	20.4 [7.6-91.3]	
Age (0-4 years)	82	65.6
BCG vaccination *		
Yes	82	65.6
No	7	5.6
Unknown	36	28.8
Contact to family member having TB	23	18.4
TB manifestation		
PTB only	64	51.2
TBM	49	39.2
Disseminated TB	9	7.2
other EPTB forms	3	2.4
HIV infection		
Positive	20	16.0
Negative	79	63.2
Unknown	26	20.8
Outcome		
Survival	97	77.6
**Withdrawn treatment	22	17.6
In-hospital mortality	6	4.8
Residence in Hanoi city	39	31.2
DST performed	121	96.8
Genotyping completed	118	94.4

IQR: Inter-quartile range; BCG: Bacillus Calmette–Guérin; PTB = pulmonary tuberculosis;

*TBM= tuberculous meningitis; DST = drug susceptibility testing; * BCG vaccination by*

*interviewing patient's parent; ** Withdrawn from treatment because of severe condition.*

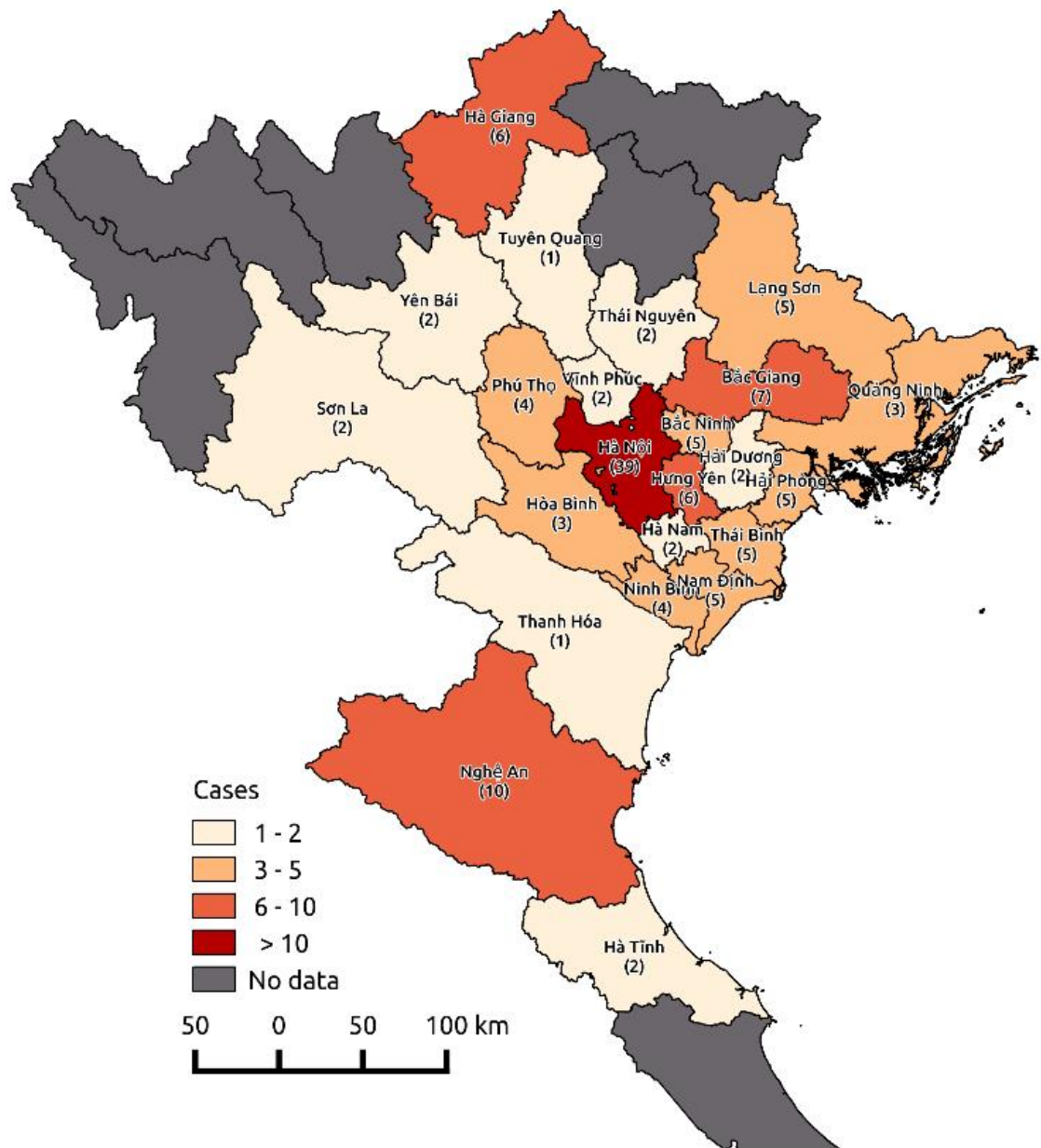


Figure 5.1 Province of residence of 125 patients from whom MTB was isolated.

5.3.2 Phenotypic drug susceptibility profiles of MTB isolates

Phenotypic DST was performed for 121 isolates (97%). In 4 isolates (3%) no DST could be done due to bacterial contamination (2 cases) or loss of viability (2 cases). The drug susceptibility profiles of all isolates are shown in Table 5.2. Of all isolates tested, 81/121 (66.9%) isolates were found to be fully susceptible to all four tested drugs. Resistance to INH was observed in 25/121 isolates (20.7%), which included 4/121 isolates (3.3%) with INH mono-resistance. Resistance to SM was detected in 34/121 isolates (28.1%), which included 15/121 isolates (12.4%) with SM mono-resistance. Rifampicin resistance was found in 4/121 isolates (3.3%). These 4 isolates were both also resistant to INH and therefore MDR. Resistance to EMB was rare, detected in only three isolates (2.5%, 3/121).

Twenty-one isolates with an INH resistant phenotypic were subjected to MAS-PCR for mutation detection (Table 5.3). MAS-PCR failed in 3 isolates with no PCR product in two attempts. Among 18 successful MAS-PCRs for detecting INH resistance mutations, all isolates had the mutation at *katG* 315 while no mutation at *inhA* was detected. Among two isolates with phenotypic resistance to RIF, the MAS-PCR results revealed one had a mutation at codon 526 and one showed absence of mutation at all positions tested (531, 526 and 516).

5.3.3 MIRU genotype distribution of MTB isolates

24 loci MIRU-VNTR typing was done for 120 MTB isolates. Based on their MIRU-VNTR types, these isolates were assigned to four major lineages (Table 5.4). Among 120 MTB isolates genotyped, the East Asian/Beijing lineage was most frequently observed (63.3%, 76/120), followed by the Euro-American lineage (16.7, 20/120). The proportion of the Beijing genotype was not significantly different among patients with PTB (38/62, 61.3%) or EPTB (37/56, 66.1%) ($P=0.70$) (see Table 5.5).

Table 5.2 Drug susceptibility profiles of 121 MTB isolates from children

Drug susceptibility pattern	No. (%)
Fully susceptible	81 (66.9)
Any resistance	40 (33.1)
Mono-resistant	19 (15.7)
SM	15 (12.4)
INH	4 (3.3)
Polyresistant but not MDR	17 (14.0)
INH and SM	15 (12.4)
INH, SM and ETB	2 (1.7)
MDR	4 (3.3)
INH and RIF resistant	2 (1.7)
INH, RIF and SM resistant	1 (0.8)
INH, RIF, SM and EMB resistant	1 (0.8)

Table 5.3 Resistance to INH and RIF of 21 isolates phenotypically resistant to INH by MAS-PCR

No	Study_ID	INH Resistance	RIF Resistance
1	NHP004	R : <i>katG</i> S315T	S
2	NHP013	R : <i>katG</i> S315T	S
3	NHP015	NA	S
4	NHP018	R : <i>katG</i> S315T	S
5	NHP024	R : <i>katG</i> S315T	S
6	NHP027	R : <i>katG</i> S315T	S
7	NHP028	R : <i>katG</i> S315T	S
8	NHP029	R : <i>katG</i> S315T	S
9	NHP031	R : <i>katG</i> S315T	S
10	NHP032	R : <i>katG</i> S315T	S
11	NHP037	R : <i>katG</i> S315T	S
12	NHP041	R : <i>katG</i> S315T	S
13	NHP046	R : <i>katG</i> S315T	S
14	NHP050	R : <i>katG</i> S315T	S
15	NHP056	R : <i>katG</i> S315T	S
16	NHP057	R : <i>katG</i> S315T	S
17	NHP059	R : <i>katG</i> S315T	S
18	NHP061	R : <i>katG</i> S315T	S
19	NHP069	R : <i>katG</i> S315T	S
20	NHP080	NA	S
21	NHP090	R : <i>katG</i> S315T	R

S: sensitive; R: resistant.

Table 5.4 Lineage classification of 120 MTB isolates from children.

Lineage	N	%
East-Asian (Beijing)	76	63.3
Euro-American	20	16.7
Indo-Oceanic	15	12.5
Unclassified	9	7.4
Total	120	100.0

Table 5.5 MTB genotype and clinical characteristics in children

Characteristics	All genotypes (N=118)	Beijing genotype (N=75)	Non Beijing genotypes (N=43)	P- value
Age median (IQR)	16.1 [6.5-72.2]	16.2 [7.0-66.0]	16.0 [6.0-87.0]	0.68
HIV co-infection	20 (16.0)	12 (27.9)	8 (10.7)	0.05
Extrapulmonary TB	56 (47.5)	37 (49.3)	19 (44.2)	0.70
Unfavourable outcome	28 (23.7)	18 (24.0)	10 (23.3)	1.00
Any drug resistance	38 (32.2)	29 (38.7)	9 (20.9)	0.06
MDR	4 (3.4)	2 (2.7)	2 (4.7)	0.62

5.3.4 Association of drug resistance with MTB genotypes

Univariate logistic regression analysis of 118 isolates for which both DST and genotype data were available showed the Beijing genotype was significantly associated with INH (OR, 3.8; 95% CI [1.2-11.9], P=0.02) and SM resistance (OR, 2.5; 95%CI [1.0-6.6], P=0.05) (Table 5.6). The number of MDR cases was too small for further analysis.

Table 5.6 MTB genotype and drug resistance in children

DR profile	All genotypes N (%)	Beijing genotype (N=75) n (%)	Non Beijing genotypes (N=43) n(%)	P-value	OR (95% CI)
Fully susceptibility	80 (67.8)	46 (61.3)	34 (79.1)	0.07	0.42 (0.2-1.0)
INH mono resistant	4 (3.4)	4 (5.3)	0	0.30	NA
SM monoresistant	13 (11.0)	8 (10.7)	5 (11.6)	1.00	0.9 (0.3-3.0)
SM and INH resistant	19 (16.1)	17 (22.7)	2 (4.7)	0.01	6.0 (1.3-27.4)
MDR	4 (3.4)	2 (2.7)	2 (4.7)	0.62	0.5 (0.8-4.1)
DR to individual drugs					
RIF	4 (3.4)	2 (2.7)	2 (4.7)	0.62	0.5 (0.8-4.1)
INH	25 (21.2)	21 (28.0)	4 (9.3)	0.02	3.8 (1.2-11.9)
SM	32 (27.1)	25 (33.3)	7 (16.3)	0.05	2.5 (1.0-6.6)
ETB	3 (2.5)	3 (4.0)	0	0.29	NA

RIF=rifampicin; INH=isoniazid; SM=streptomycin; ETB=ethambutol; MDR=multidrug resistant; DR= drug resistant; OR=Odds ratio

5.4 Discussion

As a consequence of the low rate of culture confirmation of TB among children, there are limited data on drug resistance and genotypes of MTB isolates from children although these data are thought to be useful for TB control programmes. To our knowledge, this study is one among few reports of drug susceptibility of MTB from children in Vietnam. In an earlier small retrospective study of 103 cases from northern Vietnam (287), only 14 cases had DST results available of which only one case was resistant to INH. The study reported here was performed prospectively on a total of 125 isolates collected over 5 years. This reflects the difficulty of conducting research on paediatric TB at two large referral centres in a high burden country: it took five years to collect 125 isolates from children. The findings of this study highlight the high resistance rate to isoniazid and streptomycin among children with TB in Vietnam. The MIRU-VNTR analysis also confirmed the dominance of the Beijing genotype in northern Vietnam.

The resistance rate to any anti TB drug among children found in this study was similar to the rates reported in adults. It was slightly lower than those found among adults with new TB (33.0% vs. 39.1%) as reported by Hang *et al.* in a study investigating drug resistance to first line TB drugs in Hanoi (180). Our findings are also similar to the numbers reported by the Vietnamese national TB control programme in the fourth national drug resistance survey (33.1% versus 32.7%) (179). This reflects the high rates of drug resistance among adult TB cases in Vietnam, but also confirms that these resistant isolates are actively transmitting in the community, rather than being a ‘reservoir’ of latently infected cases from the era before DOTS control in Vietnam.

The findings that over 20% of children have INH-resistant TB and that the majority of these have the *katG* S315T mutation, which confers high-level INH resistance (163, 164) raises the question whether a change in treatment policy for paediatric TB is required. The majority of children are never tested for drug susceptibility, and due to the long turnaround time required of DST, routine DST would be unlikely to inform clinical treatment decisions. The GeneXpert MTB/RIF test is only able to detect RIF resistance, and not INH resistance, at the present time, although a cartridge for INH resistance is under development. This study did not evaluate treatment outcome, and this should be reviewed before decisions are made to alter empiric treatment for children in Vietnam. WHO recommends the addition of ethambutol to the continuation phase for adults in areas with high isoniazid resistance (2HRZE/4HRE) (288) and this regimen is gradually being phased in in Vietnam. However, this regimen has been recommended based on expert opinion, without any supporting evidence from clinical trials. There is a high risk of ocular toxicity from ethambutol in young children due to the inability of children to report disturbances in eyesight, and the risk of increased adverse events in those unnecessarily treated with ethambutol is a greater concern than in adults, if this regimen is introduced as the standard regimen (289, 290). Therefore, it is unclear if a change in regimen is necessary or what the optimal regimen would be, but the data reported here clearly suggest that an evaluation of treatment outcomes is needed to determine whether the current regimen used for paediatric TB in Vietnam is effective in light of the high rates of INH resistance. Isoniazid preventative therapy (IPT) is used for exposed household contacts in Vietnam. The results found here suggest that IPT may not be the optimal prophylactic drug for use in Vietnam. The recently developed 12-dose INH-RIF regimen would also not be appropriate in this high INH-resistance setting, due to an increased risk of developing RIF resistance and therefore MDR in cases exposed to an INH resistant index case (291).

The MDR-TB proportion among children in this study is similar to that most recently reported in adults with new TB in the annual global TB report for Vietnam (43), but our numbers are small.

The findings of genotype distribution in this population are consistent with most studies among adults with TB in Vietnam with the most prevalent genotype being the East Asian/Beijing lineage followed by the Euro-American and Indo-Oceanic lineages. The Indo-Oceanic lineage is less prevalent in northern than southern Vietnam, reflecting the geographic proximity to China of the north, and to Indonesia / the Philipinnes in the south-east. This established presence of circulating Euro-American strains probably reflects the history of Vietnam, which was a French colony for approximately 100 years until the mid-twentieth century. The African lineages (lineages 4, 5 and 6) were not identified in this study and have not been reported in other molecular epidemiological studies from Vietnam (283, 285, 292-297).

The high proportion of the Beijing genotype among children (63.1%) is similar to the findings among adults in Hanoi between 2007 and 2009 (58.5% (272/465) but higher than the findings in the study by Anh *et al.*, who reported 38.5% of 221 isolates from adults in northern Vietnam to belong to the Beijing genotype between 2003 and 2004 (294). The high percentage of the Beijing genotype among children suggests the widespread ongoing transmission of this genotype in the community, supporting the hypothesis that the Beijing genotype is established and endemic in the Vietnamese population (284, 295).

An association between the Beijing genotype and resistance to isoniazid and streptomycin has been previously reported in adults in Vietnam (180, 298). This association was also found

among children in this study. Although the Beijing genotype was also reported to be strongly associated with MDR-TB in adults (284), we could not show the presence or absence of this association among children due to the limited number of MDR-TB cases in children (n=4). The underlying reasons for this association are not clear (165). Associations between DR and MDR-TB and the Beijing genotype have been reported from other locations and *in vitro* experiments have shown the more frequent acquisition of drug resistance mutations in Beijing genotype isolates (155). The availability of global WGS data for MTB is beginning to help in understanding the global distribution of MTB, the evolutionary changes this pathogen is undergoing and the evolution, distribution and epidemic spread of drug resistant MTB. However, much research remains necessary in order to fully understand under which circumstances and by what mechanism(s) the Beijing genotype is associated with drug resistance.

Our study was limited by a hospital based study design; therefore, the samples may not be representative of all paediatric TB in the population. However, the isolates were collected from most areas in northern Vietnam, and provide a preliminary insight into the population structure of MTB circulating in children, especially in view of the total absence of DST and genotypic data from Vietnamese paediatric TB population. The genetic diversity may be biased by cultivability of different genotypes, particularly in paucibacillary disease. This may also affect the drug susceptibility patterns by selective analysis of only the culture positive subgroup of strains. However, it would be expected that, if there is any effect, the drug resistant strains would be less viable and therefore less likely to be isolated in culture. This would result in an under-estimation of the true resistance rate. The correspondence between drug resistance patterns seen in adults and those described here in children suggest that there is not a strong selective effect on the paediatric subpopulation with positive cultures.

In summary, our observations in children strongly suggest a high proportion of resistance to isoniazid and streptomycin in the paediatric population and predominance of the Beijing genotype. The predominance in children suggests recent invasion and this could signal a more severe situation in Vietnam in the future in absence of more effective control.

Chapter 6

Discussion and recommendations

6.1 General conclusion and remarks

Tuberculosis is a major public health problem which causes more deaths annually than any other infectious disease (43). Although significant progress has been made in global TB control since the change of the millennium, the global research efforts and intensification of control measures has focused largely on adults. There are approximately 1 million cases of TB in children each year, and many more become latently infected every year. (53). Accurate estimates of the burden of TB among children are difficult because diagnosing TB in children is more challenging than in adults. The major reasons are the paucibacillary nature of paediatric TB and the difficulty of collecting appropriate clinical samples for testing. Therefore, research to improve TB diagnostics in children, in particular the detection yield, is important.

In this thesis, my studies have focused on the evaluation of two diagnostic methods to detect MTB in children. In addition, to better understand the epidemiology of TB in children in northern Vietnam, the drug susceptibility of TB in children admitted to the National Hospital of Paediatrics was investigated. The work described in my thesis aimed to answer the following questions.

- 1) What is the performance of MODS compared to conventional diagnostic methods in diagnosing TB in children?
- 2) Does adding mycobacterial blood culture increase the overall yield of TB diagnosis in children?
- 3) What is the prevalence of drug resistance in paediatric TB in Vietnam?

- 4) Is there an association between drug resistance and the lineage of MTB among children with TB in northern Vietnam?

The findings of my thesis provide more evidence of the need to improve diagnostics of TB in children and contribute to understanding the epidemiology of TB in children.

Performance of MODS assay in TB diagnosis in children

Chapter 3 presents the findings of MODS performance on different types of specimens at the largest general paediatric hospital in Vietnam. I show that MODS was far more sensitive than conventional ZN smear (46.0% vs. 8.8%) and had a higher sensitivity than culture on solid media (46.0% vs. 38.9%). When analysed by sample type, the study showed that the improved yield from MODS as compared to solid media can be attributed to a higher yield from gastric aspirates (27.2% vs. 19.4%). The finding of the superior sensitivity of MODS against LJ culture in this study is consistent to results from a previous paediatric study in Peru with 91% of confirmed cases detected by MODS vs. 59% of confirmed cases detected by LJ (249). In a study at the referral TB hospital in southern Vietnam, MODS showed 39.7% of sensitivity (10, 250). Compared to solid culture, the median time to positivity of MODS was significantly shorter by approximately 4 weeks (median time: 7 vs. 35 days). In addition, MODS showed a low contamination rate in comparison to that found in commercial liquid culture (2.3% vs. 7%) (104, 299).

With improved accuracy and faster time to diagnosis than solid culture, MODS is a useful liquid culture method to confirm TB cases in children in resource limited settings. It cannot be used to rule-out a diagnosis of TB. The findings in my thesis and recent studies provide strong evidence to support the implementation of MODS to improve case detection among

children in high-burden countries. During the time of these studies, considerable evidence for use of the GeneXpert MTB/RIF to diagnose paediatric was published and the test is now widely available in many urban centres in high burden countries following unprecedented scale-up of the assay. The assay has several advantages over MODS: shorter time to result (2 hours vs. several days), less biohazard risk as culture is not required and the sample reagents rapidly inactivate MTB, the ability to simultaneously detect MDR-TB, minimal hands-on time whereas MODS requires repeated plate reading by microscope. However, the GeneXpert MTB/RIF has several disadvantages: high cost (10USD per cartridge), the need for machine maintenance and continuous uninterrupted power supply while cartridges are being processed. The relative costs and benefits of each assay must be weighed carefully in making a decision as to which assay is most appropriate to a context.

During the recent years since the study was started, various modifications to the original MODS protocol have been evaluated to address the need for time consuming and tedious manual observation of MTB growth under microscopy and improve biosafety. Auto-MODS, which was recently proposed by Wang *et al.*, uses the computer assisted digital camera to inspect the MODS culture and screw-capped tubes to enhance the biosafety (300, 301). Compared to commercial liquid culture (MGIT 960), Auto-MODS had a similar MTB detection yield (95.9% of sensitivity and 97.1% of specificity) but had slower time to detection (median time: 10 vs. 6 days). However, Auto-MODS is considerably cheaper with regards to both the initial set-up (\$7,000 for MODS vs. \$85,000 for MGIT system) and individual sample cost (\$2.00 vs. \$20.00). Similarly, another improvement of MODS by using a lens free imaging system replacing the human eye was reported by Solis *et al.* This novel system is able to digitalise a 24-mm² surface with approximately 40X magnification in a single capture (302). The study findings showed that modified MODS notably reduced the

time of inspection. The interpretation of MODS results is simplified through a computer based algorithm that automatically recognises an MTB specific pattern from the digital image with a high sensitivity and specificity (99.1% and 99.7%)(303). However, it is not clear if MODS has any impact on the current paediatric TB diagnostic algorithm due to the relatively long time to result, by which time a clinical decision to treat or discharge has usually been made. The consideration about the operational feasibility of MODS comes from unfavourable findings in a retrospective review of clinical records at a primary health care centre in Peru before and after the introduction of MODS in 2008 (304), in which Mendoza-Ticona *et al.* found that universal access to MODS did not alter the final treatment outcome of new pulmonary TB in adults although it increased DST coverage and shortened the delayed time between the diagnosis of TB and receipt of DST results. Therefore, for large-scale introduction of this method into routine paediatric TB diagnostics in high burden countries, further research to evaluate effectiveness of introduction in clinical case management should be done and compared with the introduction of the GeneXpert MTB/RIF assay.

Alternative specimens for TB diagnosis in children: additional value of mycobacterial blood culture.

In chapter 4 I demonstrated that there is limited added diagnostic value of mycobacterial blood culture for TB diagnosis among children. The study was prospectively designed at both the general paediatric hospital and the referral TB hospital in Hanoi, an endemic TB setting, and recruited children with diverse TB manifestations. The findings of the study suggested that adding this test into the routine TB diagnostic algorithm may marginally increase the overall yield. However, the test performance was poor with low sensitivity and slow time to detection in comparison to routine culture. In addition, the added value of mycobacterial blood culture to the overall yield with one additional positive case compared to the high

incremental cost is too high for routine diagnosis of TB in resource limited settings. The test showed limited utility for diagnosis of TB in children in clinical context.

Recently, a study of GeneXpert MTB/RIF on blood from adults with HIV found a sensitivity similar to blood mycobacterial culture using solid culture of sputum as reference but the high volume of blood required (20ml) is not feasible for children (33). Further research to simplify blood sample concentration and to improve the detection limit of GeneXpert MTB/RIF remains needed. In addition, evaluation of GeneXpert MTB/RIF on non-invasive samples, such as stool, should be considered. The French organization, “Agence National pour Research sur la SIDA” (National Agency for AIDS Research, ANRS), has conducted a multi-country study systematically evaluating the yield of different specimens tested by GeneXpert MTB/RIF in HIV-positive children, but similar studies are needed in HIV-negative children (86). Since young children frequently swallow their sputum, MTB may be detected in the stool of children with PTB. Various studies have been performed on stool from children with PTB suspicion: the GeneXpert MTB/RIF assay detected MTB with a medium sensitivity (25–69%) and a high specificity (91.7–100%) as compared to MGIT culture (87, 305). A recent study using a new processing protocol which enables to process a larger volume of stool (up to 1.2 gram vs. less than 0.2 g in previous studies) on GeneXpert MTB/RIF showed improved sensitivity (85% [95% CI 0.6–0.9]) for TB detection in children who were positive by GeneXpert MTB/RIF on gastric aspirate (88). However, the sensitivity remains low, for clinically diagnosed TB and stool testing cannot be used to exclude a diagnosis of TB. Given the high cost of GeneXpert MTB/RIF cartridges, testing of gastric aspirate remains a better approach, unless gastric lavage is contra-indicated. Further large clinical studies are needed to evaluate the optimal processing of samples other than sputum for GeneXpert MTB/RIF.

Drug resistance epidemiology of paediatric TB in northern Vietnam

In chapter 5 in this thesis I present the drug susceptibility pattern to first line anti TB drugs among children with confirmed TB in northern Vietnam. Among culture positive patients presenting at NHP during 5 years (2009-2013), resistance to isoniazid and streptomycin is most prevalent, which accounts 1 among 4 cases. The high resistance rate to INH (20.7%, 25/121) and SM (28.1%, 34/121) found in children is similar to that reported in adults with new TB in the population (180), strongly suggesting ongoing transmission of MTB resistant to these drugs in the community. Four cases with MDR-TB were recorded, but this number is too small to draw conclusions on the rate in population. Because the study was undertaken in a general paediatric hospital setting, the findings are not representative for all circulating MTB in the paediatric population in the region. However, this is the first comprehensive report attempting to understand the DR situation in children in northern Vietnam. To better understand the trends of DR, such studies should be performed periodically, such as every 5 years, while waiting for establishment of national drug resistance surveillance. The data would provide indirect measurement of effectiveness of NTP for TB transmission control in the areas.

I also describe the genetic characteristics of MTB from children in chapter 5. The Beijing genotype was most frequently found, followed by the Euro-American genotype. This pattern is similar to that found in new TB adults in Vietnam (294, 295). These findings provide important evidence to support the hypothesis of the epidemic spread of the Beijing genotype in the population (284, 306). The Beijing genotype has been shown to increase in prevalence in a number of geographical regions following its introduction. There are no data from Vietnam, but the Beijing genotype appears to have been endemic for a considerable time and data are only now emerging which will allow the temporal monitoring of its distribution.

WGS studies allow us to identify sub-lineages, which are most actively transmitted within a region and these data will help to determine if the Beijing genotype is more virulent in the Vietnamese population than other strains currently circulating. Studies attempting to show associations between the Beijing genotype and clinical presentation or outcome have reported conflicting results and no clear picture has emerged of any correlation between Beijing genotype and clinical presentation (165). The Beijing genotype has been reported to have increased virulence in animal models for TB (307). The Beijing genotype was demonstrated to be strongly associated with any drug resistance and MDR in epidemiological studies in Vietnam, in China (308), in Indonesia(309), and elsewhere (283-286, 296, 298). In these studies, it was shown to be linked with drug resistance to isoniazid, which is among the most important first line drugs, as found in this study and various studies in adults. Although the Beijing genotype was found to be strongly associated with MDR-TB in some adult studies in Vietnam (284, 286) and other countries such as China (310), countries in Soviet Union, there was no observation of association of this genotype with MDR among children in this study but this was due to small numbers. A study with large sample collection of MTB from children at national level would reveal whether this association exists in the population.

6.2 Recommendations for future research

Several calls to action on paediatric TB in the last decade have drawn attention to the neglected issue of paediatric TB and have led to some important advances. WHO now collects age-disaggregated data on paediatric TB and encourages national TB programmes to address paediatric TB as a strategic priority. Many countries, including Vietnam, have now revised National TB data collection to systematically collect data on paediatric TB.

In 2015 new paediatric drug formulations of the first line TB drugs were made available for the first time to facilitate correct dosing in children but action is still needed to expand access.

Drugs previously had to be administered by splitting or crushing adult tablets, making precise dosing impossible. Further, the fixed dose combination tablets used by most national TB programmes are also of the wrong ratios to allow correct paediatric dosing according to the latest guidelines. These new formulations are a major advance in improving treatment of paediatric TB.

New approaches to TB diagnosis have been systematically evaluated in children, with some successes and failures. The GeneXpert MTB/RIF test has been shown to be an effective test for diagnosing TB with high specificity in children, but sensitivity is low. This remains true for all microbiological and molecular tests for TB in children. The GeneXpert MTB/RIF test was endorsed by WHO for diagnosing TB in children following systematic review of the evidence in 2013(127). Despite these advances, there remain many urgent priorities for paediatric TB research.

The findings of this thesis highlight some gaps in our current knowledge and reinforces the need for further study in order to improve the performance of current diagnostic tests for TB confirmation in children as well as to better understand TB drug resistance in children.

TB diagnostic study in children

MODS was endorsed by WHO and recommended for use in limited resource settings since 2010. So far, direct evidence of utility of this test for TB detection in children including our research is limited, especially there is a lack of evidence as to whether MODS can be used for direct DST with paediatric specimens. The following research priorities should be addressed:

- ✓ Investigate the performance of MODS for direct drug susceptibility testing in paediatric specimens, including second line drugs.

- ✓ Evaluate the cost effectiveness of MODS in case management, especially in general paediatric hospital and compare with GeneXpert MTB/RIF.

Culture should be performed for all samples but takes at least one week, even for the most rapid liquid culture tests. In this study, MODS took a median of 7 days to return a positive result. Therefore, rapid molecular tests like GeneXpert MTB/RIF should be the initial test of choice, especially for severely ill children in whom prompt diagnosis and treatment is essential. A culture should still be performed alongside GeneXpert MTB/RIF as the yield is higher, and an isolate is necessary for full DST. Published MODS research evaluates mainly respiratory samples and only CSF for EPTB. Evidence of GeneXpert MTB/RIF on readily obtained samples like blood, urine or stool has shown disappointing sensitivity. Several factors are likely to contribute to this, but the most important is the low bacillary load in such samples, which is likely to be addressed by increasing sample volumes and concentrating bacilli prior to inoculating the GeneXpert MTB/RIF cartridge. Other factors include inhibitors to the PCR, which changes in sample processing may help to eliminate. Cepheid have announced the imminent release of a new GeneXpert MTB/RIF cartridge, called GeneXpert MTB/RIF Ultra which is claimed to be more sensitive than the previous version (311). This has been achieved by increasing the DNA targets and increasing the sample volume added to the reaction chamber, among other modifications. This new cartridge may substantially increase case yield in paediatric TB, and it is hoped sensitivity will approach that of culture. Multi-site, multi-country standardised evaluation of GeneXpert MTB/RIF Ultra in paediatric TB diagnosis is a priority. Further studies on GeneXpert MTB/RIF should be included as follows:

- ✓ Develop and evaluate optimised specimen processing methods for stool, urine and other EPTB samples for GeneXpert MTB/RIF .

- ✓ Evaluate the accuracy of GeneXpert MTB/RIF Ultra vs. GeneXpert MTB/RIF for TB diagnosis in children

Testing of multiple samples has repeatedly been shown to increase the overall yield of detection. The inclusion of blood samples in the study described in chapter 4 appeared to increase the overall yield, albeit only marginally. Other samples (such as urine, stool) were not examined in this study due to resource limitations, but the optimal specimen collection protocol (sample type and volume) for paediatric TB should be systematically elucidated.

- ✓ Evaluate other specimen collection protocols to determine the optimal combination of sample types and volume to maximise diagnostic yield of TB in children.

Biomarkers in blood, urine and sputum have been systematically researched for three decades to try and devise an optimal diagnostic point of care test. Initial research focused on individual candidate biomarkers, but tests failed to demonstrate sufficient sensitivity, specificity, or often both. Recent approaches have used more sophisticated analytical techniques, such as MALDI-TOF and SELDI and attempted to identify combinations of biomarkers, or ‘signatures’, which identify TB cases (62). Jenum *et al.* showed the association of 12 immune biomarkers with the likelihood of TB disease among children. This attracts further research to exploit the potential of the immune markers for diagnosis TB in children but is unlikely to result in a point-of-care diagnostic in the next five years.

Tuberculosis epidemiology in children

To understand the epidemiology of TB in children, long-term research should be conducted to see the trends of incidence, which would reflect truly the transmission in the population. Because most studies on childhood tuberculosis resistance prevalence are based on isolates, which are rare in children, the findings may have selective bias and not reflect the true situation. As molecular detection becomes more sensitive, it should be possible to detect drug

resistance patterns by whole genome amplification from single bacilli in clinical samples. Several authors have reported successful whole genome sequencing of MTB from sputum samples, but not yet from children and it is likely that significant improvement in sensitivity will be required before this is possible. To better assess the drug resistance epidemiology and diagnosis in children, research to evaluate the optimal approach among existing methods should be a priority.

Finally, treatment of TB in children has not been considered in this thesis, but the high rates of drug resistance in children in Vietnam shown in this study, highlight the urgent need for inclusion of children in trials of new drugs and drug combinations. Alongside these studies, PK studies must be conducted on children to ensure optimal dose and formulations are identified.

The END TB strategy (253) has set an ambitious global target of TB elimination as a public health problem by 2035. This could potentially be achieved with sustained political and social commitment at all levels, but an essential component will be research leading to better drugs, diagnostics and an effective vaccine. Children must be included in this research at all levels to ensure this vulnerable population benefit from advances in TB diagnosis, prevention and management in tandem with the adult population. If these ambitious plans can be realised, children born today will see the consequences of TB removed from their children's lives. If we fail to control drug resistant TB now, these children may see a world where TB is once again untreatable with chemotherapy and surgery and sanatoria are our only weapons.



ENDING TUBERCULOSIS IN CHILDREN

KEY FACTS



1 MILLION
children fell ill with
TUBERCULOSIS
(TB) in 2014

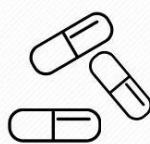


400
children die
each day
from TB

CHALLENGES IN REACHING CHILDREN WITH TB



DIFFICULTIES IN
TB DIAGNOSIS
lead to children often
getting missed or
overlooked



TB TREATMENT NOT
CHILD-FRIENDLY
leading to poor health
outcomes and development
of drug-resistance



INCREASED
COLLABORATION NEEDED
between actors in
TB, HIV and maternal
and child health

http://www.who.int/tb/childhood_TBinfographic.jpeg

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Appendix A

Evaluation of Microscopic Observation of Drug Susceptibility (MODS) technique for the diagnosis of Pediatric Tuberculosis at National Pediatric Hospital, Hanoi , Vietnam

Introduction

Pediatric pulmonary and extra-pulmonary tuberculosis (EPTB) requires rapid diagnosis and instigation of appropriate chemotherapy to improve outcome. The current options for rapid diagnosis of tuberculosis in developing countries are smear for acid fast bacilli (AFB) or PCR. Solid cultures techniques are more sensitive, but slow and hazardous. EPTB is often AFB smear and culture negative, and smear-negative and EPTB are more common in children compared to adults.(4) Furthermore, it is challenging to obtain adequate specimens for TB diagnostic tests from children, for instance many children are unable to produce sputum.(1) Thus, there remains a great need for new rapid diagnostic tests for pediatric TB.(5)

The Microscopic Observation Drug Susceptibility (MODS) assay is a rapid culture system that allows early identification of TB and drug susceptibility testing.(6) The latter is a great advantage, as AFB smear and PCR do not allow for the microbiological isolation of *M. tuberculosis* and assessment of drug resistance.

MODS

MODS is a rapid liquid culture system that is performed in standard tissue culture plates and is read with an inverse light microscope. The technique allows the early identification of *M. tuberculosis* from clinical samples through recognition of the characteristic cord formation of *M. tuberculosis*. The rapid microbiological isolation of *M. tuberculosis* also allows for the subsequent drug susceptibility testing of the isolate and rapid identification of MDR TB. Without second line therapy, MDR TB is almost always fatal.(3) Also further transmission of MDR TB can be prevented.(3)

A recent study in adults showed that the sensitivity of detection of positive sputum cultures was 97.8% for MODS culture, 89.0% for automated mycobacterial culture, and 84.0% for Löwenstein–Jensen (LJ) culture, with a median time to positivity of 7 days, 13 days, and 26 days, respectively.(6) Also the median time to drug susceptibility results was faster for MODS as compared to automated and Löwenstein–Jensen culture: 7, 22, and 68 days, respectively. Furthermore, MODS is inexpensive, simple and applicable in resource-limited

settings, like Viet Nam (2). Another advantage is that the MODS assay is a closed system, which makes it a safer technique for laboratory workers than standard TB culture techniques.

A good evaluation of MODS technique for the detection of pulmonary and EPTB in a pediatric setting in an endemic country is still lacking. One study on pulmonary TB has been performed in Peru, with similar results as the study in adults: better sensitivity and faster than standard culture techniques.(7) MODS has not been evaluated for extra-pulmonary TB in children. It has been evaluated for the isolation of *M.tuberculosis* from CSF in case of suspected tuberculous meningitis in adults at the Hospital of Tropical Diseases, Ho Chi Minh City, Vietnam (Maxine Caws et al. manuscript submitted) and is shown to be moderately sensitive (65%), specific (100%), with a median isolation time of 6 days. Both for pulmonary and EP TB, MODS is considered to be a potential valuable diagnostic test, that needs further evaluation.

This study aims to evaluate the diagnostic value of MODS for both pulmonary and extra-pulmonary TB in children admitted to the National Hospital of Pediatrics (NHP) in Viet Nam.

Objectives:

This project will aim to:

1. Enhance the overall diagnostic capacity at NHP to diagnose childhood tuberculosis. Currently, the microbiology laboratory of NHP only performs PCR on diagnostic specimens. For culture, the specimens are transferred to the laboratory of the National Institute of Tuberculosis and Pulmonary Disease.
2. Set up laboratory capacity to perform MODS technique for diagnosis of pulmonary and extra-pulmonary tuberculosis in children admitted to NHP.
3. Compare MODS with conventional AFB smear, PCR and TB culture, through a lab enhancement program of children who attend NHP during a period of 2 years and are suspected of having any form of TB.
4. Set up TB research projects, once MODS has been well established at NHP.
5. Epidemiology of TB drug resistance in children admitted to NHP.

Study design:

Pediatric patients admitted to NHP with suspected TB, both pulmonary and extra-pulmonary, are evaluated with MODS, AFB smear, PCR, and Lowenstein culture. The latter will be performed at the National Institute of Tuberculosis and Pulmonary Disease.

Specimens that will be tested by MODS diagnostics

Specimens from patients presenting at NHP with a clinical suspicion of any form of TB, will be eligible for enrollment.

Doctors will be trained to sample adequate specimens from suspect TB cases. Suspect TB cases in this study are considered to have **at least two** of the following findings:

- unexplained fever for more than 1 week
- unexplained cough for more than 1 week
- radiographic findings suggestive of tuberculosis.
- failure to thrive/weight loss
- enlarged non-tender lymph nodes or lymph node abscess, especially of the neck
- signs of meningitis with history of at least one week
- HIV positive
- malnourished
- TB contact history
- treating doctor suspects TB based on findings not described above.

As MODS can be considered as routine lab test, no informed consent will be requested from the patient or legal guardian(s). Patient data will be anonymized.

Exclusion criteria for specimens to be tested by MODS

The following specimens are excluded:

- specimens from a patient that is on anti-tuberculous treatment

Definitions

Pulmonary tuberculosis

Proven

- microbiological detection of TB in respiratory or gastric specimen and pulmonary symptoms by AFB smear, PCR, or LJ culture.

Tuberculous meningitis:

Proven:

- microbiological detection of TB in CSF by either AFB stain, PCR, or LJ culture
- clinical meningitis, CT scan is consistent with TBM (basal meningeal enhancement, tuberculoma, oedema) and clinical response to antituberculous treatment. No other pathogens detected.

Non - TBM:

- CSF is positive for other pathogen
- Sustained clinical improvement without anti-tuberculous treatment.

For all other causes of pediatric tuberculosis:

Only microbiological (AFB smear, PCR, LJ culture) confirmed TB is considered as 'proven'. If otherwise, TB is not considered to be a cause of the patient's clinical condition.

Discrepancies

In case of discrepant results, one test being positive and other is negative, it will be assessed whether the patient had symptoms that fit the diagnosis of tuberculosis. If so, the positive test will be considered to be 'confirmed' by the clinical presentation.

Clinical Data Collection

Basic demographic, presenting clinical features, TB contact history, BCG and vaccination history. HIV testing will be performed according to hospital practice. It is good clinical practice to test suspected TB patients for HIV. Patient data will be anonymized and entered into a database for further analysis.

All patients Laboratory Diagnostics

Sample collection

Training will be given to clinicians on the indications and taking of appropriate samples for TB diagnosis:

1. In case of suspected pulmonary tuberculosis, the following specimen types are encouraged to obtain:
 - morning sputum collected on two consecutive days (spot-morning-spot)

- if an adequate sputum sample cannot be obtained, a gastric aspirate is performed on two successive mornings.
 - other samples (sputum/broncho alveolar lavage/pleural fluid/biopsy) will be taken as clinically indicated.
2. In case of suspected TBM, cerebral spinal fluid will be obtained, according to hospital guidelines. It will be encouraged to obtain large volume as possible in the children.
 3. Samples for extra-pulmonary tuberculosis will be obtained as clinically indicated.

Sample size.

On a yearly basis, around 350-400 specimens for TB diagnostics are received by the NHP microbiology laboratory. Therefore, it is expected that 750 specimens from approximately 600 patients will be enrolled in this study. The estimated TB prevalence in our study population is estimated to be 20%, resulting in 120 positive cases in two years. At an estimated sensitivity of MODS to detect pulmonary TB of 90%, the confidence interval would be $\pm 6.4\%$.

Residual specimens

Residual specimen following laboratory investigations will be stored at -80°C for further microbiological assessment in the event of contamination

Forms and data handling.

All data concerning the study will be recorded prospectively and will be transferred to a computerised database. Data will be anonymized.

Ethical considerations.

As this study will evaluate a routine diagnostic test, no Oxford University (OXTREC) approval will be obtained. The protocol will be submitted to the Review Board of NHP for local approval. All patient data will be anonymized for data entry.

Publication

Publication will be in international peer-reviewed journals following relevant guidelines. No publication will be submitted in any language without prior approval of the involved investigators.

Budget

The Oxford University Clinical Research Unit (OUCRU) will meet the costs of procedures additional to routine care for study participants as agreed with the participating hospital.

A room in NHP microbiology laboratory will be refurbished and equipped for MODS culture. Benching and equipment will be provided by OUCRU.

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Appendix B

Enhancing diagnostics for paediatric tuberculosis by blood culture

Summary

Detection of *M. tuberculosis* in clinical specimens of children has a low sensitivity because specimens are either difficult to collect or contain low levels of *M. tuberculosis*. Diagnostic criteria are non-specific and culture confirmation is challenging, as sputum samples are not often obtainable from small children and specimens typically have low yield. One is often forced to begin empiric treatment or treatment is delayed causing harm and risk to patient and contacts. The emergence of drug-resistant TB into the pediatric population demands greater attention to improved diagnostics and drug susceptibility testing (DST). Although children are typically thought to have paucibacillary disease, they are at greater risk for dissemination of TB. This may allow for detection of *Mycobacterium tuberculosis* from other bodily fluids than sputum or gastric aspirate, including blood and urine. Unfortunately, little is known about the overall yield from these various specimens. From pilot data collected among adults and children in Tugela Ferry, we know that it is feasible to collect and test various bodily fluid specimens for TB culture. Also among HIV-infected patients suspected to have extrapulmonary TB, approximately 33% have positive blood cultures for *Mycobacterium tuberculosis* when using the Bactec (automated liquid media) system.

All children ≤ 15 years old suspected of active TB presenting to the National Hospital of Pediatrics (NHP) or National Lung Hospital (NLH), Hanoi, Vietnam, will be consecutively enrolled by trained staff and those eligible will have gastric aspirate, expectorate (if possible), blood and urine collected for mycobacterial culture to study the yield in this population. All specimens (sputum, gastric aspirate, blood and urine specimens) will be tested at the microbiology laboratory. Sputum and gastric aspirate will be digested and decontaminated by the N-acetyl-L-cysteine (NALC)-NaOH method. An aliquot will be used for microscopic sputum examination for AFB (Ziehl-Nielsen-stained) and the remainder will be cultured by routine mycobacterial culture technique. Urine will also be tested by a TB antigen test (LAM Test). Blood cultures (MycroF/Lytic) will be incubated for 42 days.. Drug susceptibility testing of all positive culture will be performed using molecular techniques (HAIN test). Culture results will be provided to the treating clinician immediately upon becoming available.

Hypothesis

We hypothesize that blood cultures will detect *Mycobacterium tuberculosis* from children suspected of disseminated TB, and that a proportion of these non-sputum bodily fluids will detect both drug-susceptible and drug-resistant tuberculosis when sputum or gastric culture does not.

Primary aims

Among children (≤ 15 years) suspected of active *Mycobacterium tuberculosis* presenting to NLH and NHP, Hanoi, Vietnam:

- Assess the diagnostic yield of TB-blood culture as compared to direct smear and TB culture of expectorate and/or gastric aspirate and antigen test of urine.

Secondary aim

- Develop a diagnostic algorithm for suspected pediatric tuberculosis

Background

Tuberculosis (TB) is a major cause of morbidity and mortality among children in developing nations [1]. The HIV epidemic has fueled a resurgence of TB globally [2]. Worldwide, approximately 10% of incident TB cases are thought to occur in children but in endemic areas, the proportion rises to an estimated 40% [3]. As concerning as the outbreak is in adults, further worry is that the diagnosis of TB is difficult in children [5]. Symptom-based diagnostic criteria are non-specific and culture confirmation is challenging, as sputum samples are often believed to be too cumbersome to obtain from small children and specimens typically have low yield due to the paucibacillary nature of pediatric TB. Culture confirmation may be obtained in as few as 10% of cases of suspected pediatric TB. For these reasons, the true extent of the (drug-resistant) TB epidemic in children is unknown. Thus, either clinicians begin empiric treatment without diagnosis or no treatment is given at all. Current laboratory methods, if available at all in resource poor settings, employ smears from expectorated sputa or gastric aspirates which have low sensitivity in children [3]. While more rapid diagnostic techniques have been developed, there is still poor sensitivity in children. PCR based tests, for instance, perform well in smear positive samples, and can offer first-line drug susceptibility testing (DST). Similarly, a low-cost test, the Microscopic Observation Drug Susceptibility (MODS) assay was thought to be promising for diagnosis of pediatric tuberculosis [6,7] but work with the MODS assay among adults with suspected TB from the same community in Tugela Ferry have yielded differing results from published studies. Improving the diagnosis of pediatric TB must focus on better efforts, including more aggressive strategies to uncover disseminated disease.

A single sputum induction has recently been demonstrated to have an equivalent culture yield to three gastric aspirates for the diagnosis of pediatric tuberculosis from a referral hospital in South Africa [8]. Despite the study's findings, criticism has been targeted at the ability to perform adequate sputum induction at the rural or district level. Currently, gastric aspirate remains in the national practice guidelines as the preferred means of culture confirmation for children unable to expectorate.

Culture confirmation of disseminated disease can be obtained from blood, urine, cerebrospinal fluid (CSF), peritoneal and pleural fluid, or purulent material from lymph node aspirates, abscesses or otorrhea. Unfortunately, little is known about the overall yield from these various specimens in children. Furthermore, among HIV-infected adults suspected to have extrapulmonary TB, approximately 33% have positive blood cultures for MTB when using an automated liquid culture system [9].

Although WHO guidelines encourage body fluid collection in order to make a diagnosis of TB in children, at present, blood and urine cultures are not obtained for mycobacterial culture. However, this study seeks to demonstrate that routine investigation of blood and urine will augment the yield of traditional sputum culture for children in whom disseminated disease is more likely. Improved culture confirmation will allow DST and a more accurate description of the drug-resistant TB epidemic for children in the region.

Inclusion criteria

- Aged 0-15, presenting at NLH and NHP
- Any form of TB suspected based on **at least two** of the following findings:
 - unexplained fever for more than 2 weeks
 - unexplained cough for more than 2 weeks
 - radiographic findings suggestive of tuberculosis.
 - failure to thrive/weight loss or malnourished
 - enlarged non-tender lymph nodes or lymph node abscess, especially of the neck
 - signs of meningitis with prodromal stage of at least one week
 - HIV positive
 - TB contact history
- Relevant material (sputum or gastric aspirate, blood, and urine) available for microbiological diagnosis.
- Informed consent obtained from the patient's legal guardian(s).

Exclusion Criteria

- Age >15 years
- Diagnosed or treated for TB in the past year, received drugs effective against TB in last 3 months.
- Clinical contra-indications to collect the required study specimens

If patients are excluded, the patient will be thanked for their time and the form should be kept together with all other enrolled patient forms. This will be important at the end of the study to track how many patients were screened and reasons for exclusion.

Definitions

Detection of *M. tuberculosis* from biological specimens

Not TB

Sputa, blood or relevant specimen negative by AFB smear, and culture for *M. tuberculosis*, with either a resolution of clinical symptoms and radiographic abnormalities following antibiotic therapy not involving drugs active against *M. tuberculosis*, or with an alternative diagnosis

Clinical Data Collection

Basic demographic, presenting clinical features, TB contact history, BCG and vaccination history. HIV testing will be performed according to hospital practice. Patient data will be anonymized and entered into a database for further analysis (see data management section).

Sample size and analysis

On a yearly basis, around 400 specimens for TB diagnostics are received by the NHP microbiology laboratory (specimens from NLH are added to reach the sample size since mid 2012). Therefore, it is expected that 800 specimens from approximately 600 patients will be enrolled in this study. The estimated TB prevalence in our study population is estimated to be 20%, resulting in 120 positive cases in two years. At an estimated sensitivity of blood or urine to detect disseminated TB of 25%, the confidence interval would be $\pm 6.4\%$.

Patient enrollment

Overview: All children age 0-15 years presenting to NHP or NLH thought to have TB infection according to inclusion criteria listed in this protocol are eligible for the study. In addition, any child with failure to thrive or if the clinician caring for the child has a strong clinical suspicion for TB, then the child will also be eligible for enrollment.

General Procedures: Subjects will be enrolled Monday through Friday at NHP and NLH. On Monday through Friday mornings the study coordinator will contact the head of departments to discuss if any new admissions are TB suspects. The study coordinator can be contacted directly by any referring physician during business hours Mon-Fri. The study coordinator will also make periodic visits to the in- and outpatient departments to remind physicians about the study.

If the patient is eligible and all necessary consent/assent forms have been signed, then the sample collection procedure will start. It is estimated that 10 pediatric patients are TB suspects at NHP and NLH per week, and the goal will be to enroll all eligible pediatric suspects.

General Patient Interview Form: All suspects will be screened for enrollment by trained hospital staff using the form titled, General Patient Interview Form. This form needs to be completed on all screened patients. The first page contains patient identifiers (e.g., name, address, telephone number) and will be recorded on the cover sheet only, which will be separated and kept in a locked filing cabinet; only the study ID number should be used on each subsequent page General Patient Interview Form and placed in the upper right corner using a pre-printed label.

Parent/Guardian Consent Form: This form is to be read and explained to the parent/guardian of the patient in Vietnamese. The form should be signed by the parent/guardian and the research assistant who has obtained the consent. The consent form will be stored together with the first page of the General Patient Interview Form and will be separate from the other patient data forms to maintain confidentiality. If the child is an orphan an appointed social worker can serve as temporary guardian for the child and sign the Parent/Guardian Consent Form.

It is important for parent/guardians to understand all sections of the consent form, so as much time as is needed will be spent to clearly explain and answer questions. Key sections of the consent form to ensure the parent/guardian understands are: Procedures, Risks and Benefits, Alternatives, and Confidentiality. A copy of this form should be given to all parent/guardians.

Patient procedures

Patient fulfilling the inclusion criteria for suspected TB will be informed of the study. For those patients that assent and whose guardians provide consent samples will be collected as described below at the time of assessment and before a diagnosis is made or treatment commenced. Clinical assessment for TB will be performed by hospital staff according to normal procedures. All laboratory results will be returned to the patients doctors at the earliest possible time to assist in diagnosis. HIV testing will be

performed if informed parental consent is given. Participating patients will be assigned a study number. Patient names will not be included in the data base.

Samples

Samples for routine diagnosis

- 2 spontaneous sputum samples or 4ml gastric aspirate (spot, morning, spot) for concentrated smear, and liquid culture. .
- 2-6 ml of CSF if TBM suspected for concentrated smear and liquid culture.
- 3-5 mL blood sample will be taken in a MycoF-lytic blood culture bottle.
- 5 mL of morning urine for antigen test Determine® TB-LAM (Alere, USA)

Training will be given to clinicians on indications and appropriate collection of samples

Patient Procedures

Overview

Once patients are determined to be eligible for the study, and all forms described in the Patient Enrollment section are completed, subjects must submit samples for blood and urine. In children 8 or older that are able to expectorate, they will have sputum collected. In children 7 years of less or any child unable to expectorate, they will have sputum induction performed. Sputum induction will be preferentially invasive for children less than 5 years old. Additionally, in the subset of patients admitted to the ward and less than 5 years old, two morning gastric aspirates will be collected. All samples must then be clearly labeled and dated with the patient's unique study number labels and stored for transport. A study specimen register will be completed each day by the study coordinator. Following collection of the last specimen, the patient's participation in the study is complete. If a patient has another body fluid sent for mycobacterial culture aside from sputum, gastric aspirate, blood or urine by the treating physician during routine course of care, the result of the culture for that body fluid will be recorded, but no active collection of any other body fluid will take place for study purposes.

Assigning a study number

Once a patient is determined to be eligible for the study and all forms are completed, the patient must be assigned a study number. Study numbers and labels will be pre-printed and can be obtained from the study coordinator. A study number will be assigned in a sequential

manner by hospital , based on order of enrollment (the first patient enrolled at NHP will be MYC001, the second patient will be MYC002, etc. and for NLH the first patient will be NLHMYC001, and so on)

Once a sheet of labels is obtained with the patient's study number, enter the date of enrollment on the space indicated (date of each sample collection will also be entered for labels affixed to each sample container, and this date will differ from the date of enrollment for some samples. Study coordinator will need to assure that labels are affixed to the following:

- Every page of the General Patient Interview Form and Specimen Test Request Form
- Every sample collection bottle/tube with added date of collection and volume of collected sample

Blood Collection

For all subjects enrolled in the study, the nurse will collect between 3-5ml of blood under sterile conditions and inoculate the blood into a Bactec (MycoF-Lytic) mycobacterial culture bottle. The bottle will be labeled and transported to the laboratory at NHP within a day to be incubated in the Bactec 9050 automated system.

Urine Collection

Urine collection follows routine procedure to collect sterile urine. For subjects admitted to the hospital, first-morning, clean-catch urine samples will be obtained by the nurse for those who are able to void on command. For patients too young to void on command, an early morning urine specimen will be collected by securing a sterile plastic bag to the perineal area after sterilizing the skin (Optional). At least 5ml will be collected. Collection cups will be labeled with the study number and sent to laboratory for testing and storage.

Gastric Aspirate

For subjects admitted the hospital, the nurse will perform early morning gastric aspirates on at least two separate mornings. Gastric aspiration will be performed by NHP and NLH protocol, with attempt to withdraw at least 4ml of gastric fluid, accompanied by instillation of NaCl if necessary to produce adequate sample volume. Collection bottles will be labeled with the study number prior to transport to laboratory for testing and storage.

Sputum Collection

All subjects enrolled in the study will have sputum collected. Those unable to produce adequate sputum samples will only have gastric aspirate performed. The sputum sample and Specimen Test Request Form will be sent to the laboratory for testing.

Laboratory procedures

Specimen arrival: Specimens will arrive daily Mon-Fri, before 1 pm.

Specimen processing: Sputum and gastric aspirates will be decontaminated using the NALC-NaOH method and processed for AFB microscopy (ZN) and culture . Urine will be also tested with Determine® TB-LAM (Alere, USA) to detect TB antigen. Blood cultures will be incubated and read daily up till 42 days. HAIN test will be performed at OUCRU lab on all positive cultures to confirm TB identification and resistance to isoniazid and rifampicin.

Reporting Results

All results of the specimens will be used for clinical care. Therefore it is essential that the laboratory manager will be responsible for assuring the timely and accurate reporting of AFB microscopy and culture results. Results of AFB microscopy and any positive culture result will be reported within 24 hours of the receipt of that result in the laboratory. Negative growth for liquid culture will be reported by week 4 after day received in laboratory and blood culture by week 7. If contamination exists or another reason for re-plating occurs, at week 7 a report will be sent that culture work is still in progress and results are pending.

Storage of Isolate

All isolates of positive TB cultures will be labeled and stored in secure freezers for possible future investigation.

Data management

Overview: The data manager will be responsible for coordinating all data received from NHP and NLH patient enrolment and lab results. These results will be coordinated in an electronic Peds TB Diagnosis Study Register (for purposes of patient care), paper printouts (also to be distributed for patient care to physicians, site of enrolment and treating physicians), and an anonymized Microsoft Access Database.

Peds TB Diagnosis Study Register: The study number, specimen type (sputum, blood etc.), specimen date of collection, and patient demographic information will be entered in this register by the study coordinator. This information will be password protected. Once test results become available, the data capturer will include AFB microscopy, culture and DST

results into the study register. This register will be periodically printed and given to the participating departments for patient care purposes.

Microsoft Access Database: The data capturer will enter all data from the study register and patient forms into the Access database on a weekly basis. The information contained within the Access database will be devoid of all patient name and contact information and will not be used for clinical care.

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STUDY PERSONNEL

Overview: The Pediatric TB Diagnosis Study will be conducted at NHP and NLP

The study will be carried out by professionally trained study personnel who will be responsible for ensuring that all aspects of the project, from patient enrollment and specimen collection, to the reporting of results and data management, adhere to the protocol as approved by ethics committees at NHP, NLH, and Oxford University.

The study will be overseen onsite by Dr. Heiman Wertheim. The laboratory aspects will be overseen by Dr. Hung (NLH), Dr. Hang (NHP), Ms Sinh (study coordinator of NHP), Dr Van (study coordinator of NLH), Ms Linh (study coordinator of OUCRU).

Study coordinator: Responsibilities in this capacity include ensuring adequate weekly patient enrollment (target: 5-10 patients/week), proper labeling of all study samples and forms, ensuring that all forms are completed in full, coordinating transport of samples, and coordinating delivery of study results for data entry. The study coordinator will remain in direct communication with investigators from OUCRU via email and weekly telephone conference calls.

Data management (Oxford staff): 1-2 data managers will be responsible for data entry. Responsibilities for the project include updating and entering data from the enrollment forms into the Microsoft Access Database. The data capturer will also work with the study coordinator to ensure that all forms are complete, and that all information needed for each patient is available.

Laboratory technicians: 2-3 laboratory technicians will be assigned to process the samples for sputum, gastric aspirate, blood and urine (and occasionally other specimens).

Appendix C

Participant information sheet for participation in ‘improve TB diagnosis study’ for parents or legal guardians

Patients admitted to the National Hospital of Pediatrics who are suspected of having tuberculosis are being asked to join a research study of improving the diagnosis of tuberculosis by testing blood. About 800 children will be asked to join the study. Participating in this study is your free choice. No one can force your decision. Please take your time to read this information before you decide. If you cannot read it, someone will read it for you. The person reviewing this form with you will answer any questions you have. If you are willing to have your child join this study you will be asked to sign or place your mark on the last page.

Why is this study being done?

Your child is suspected of having tuberculosis. Often it is very difficult to confirm this diagnosis by laboratory testing. In general, a sputum sample is tested by in the laboratory, but even if your child has tuberculosis, this test can be negative. Therefore it is important to find better tests for children who may have tuberculosis. It is known that children can have tuberculosis in body parts other than the lungs. Therefore we may be able to test for tuberculosis in the blood or urine. This study will collect blood and urine to test them against the regular tests to see if they can give a better test result.

Who is doing this study?

The research team has doctors from the National Hospital of Pediatrics and the Oxford University Clinical Research Unit in Hanoi.

What will happen to your child in this study?

Whether or not your child joins the study s/he will receive standard hospital care for her/his condition. This usually involves being examined everyday and laboratory testing decided by your treating doctor. For this research study you will have all of the routine tests decided by your doctor and you will also have samples taken of stomach fluid , sputum, blood and urine soon after the doctor suspects tuberculosis to be the cause of the symptoms.

The stomach fluid is taken by inserting a tube through your child's nose and passing it down into the stomach. After the nurse has checked that the tube is in the right position, 10 mL of gastric fluid will be collected using a syringe connected to the tube. This procedure will be done twice on two separate mornings. This procedure is a part of regular care at the National Hospital for Pediatrics. Blood will be collected by inserting a needle into a vein. With a syringe the nurse will collect at least 3 mL and up to 5 mL. This blood is injected into a special bottle for testing. 5 mL of urine will be collected in a container at a time that your child has to urinate. On three different occasions, sputum will be collected by coughing lung fluid into a container. Sputum collection in the morning is the best time. It is possible that your child has signs of tuberculosis at other body sites, like the brain or abscess. In such cases, the doctor will collect samples as they normally would for regular care. All specimens (sputum, stomach fluid, blood, urine, or routine collected specimens for TB testing) will be tested at the laboratory of the National Hospital for Pediatrics.

What will happen to the samples?

These specimens will be tested for mycobacteria which can show if your child has tuberculosis. If you agree, samples collected as part of this study and also samples taken during your hospital stay to look for bacterial infection will be stored up to five years.

Why are we asking to store samples?

We want to store your child's samples because some of the tests will not be done while you are in hospital. If other samples are taken which your doctor thinks are important for your treatment e.g. the fluid from your lung or more blood, we will also store the left over from these samples so they can be tested.

One test we may want to do in the future is to see if certain people are more likely to develop infection compared to other people; this is called genetic testing. You can choose not to have this genetic testing done by marking at the end of this form

What is the benefit of participating?

You may benefit from the study because extra tests are being done which may tell your doctors about your illness and how to treat it earlier than usual.

What are the risks of being in the study?

This study involves sample collection that is done routinely in this hospital. The risks for each sample are:

-Stomach fluid collection. This test is already done regularly at NHP to test for tuberculosis. Minor problems can include nose bleed and discomfort. More significant problems include breaking the tissue in your throat or taking fluid from the lungs. These problems are rare when the samples are taken by experienced staff. In case such a problem may occur, your child will be treated for the problem.

-Sputum collection. There are no risks related to this procedure.

-Blood collection. Blood will be collected by inserting a needle into a vein. This can cause discomfort and anxiety with your child. Other complications can be bruising at the site where the needle was inserted.

-Urine collection.. No risks are known for this procedure.

What are the costs for being in the study?

There will be no additional costs to you if you participate in this study. You will still be responsible for covering the costs of your regular care but the research team will cover all the costs of tests done as part of the study.

What happens if I decide not to be in the study or change my mind later on?

This is no problem. The doctors will respect your decision. No one can force you to join the study or to remain in the study, if you change your mind later on. Your regular treatment will not be changed because of your decision.

Can someone else decide to stop the study?

The research team doctors can stop your child being in the study if they think his/her health may get worse because of the study or if your child does not follow the study procedures. The sponsor or the Ethics Committee that reviews this study can also decide to stop the study.

Confidentiality

All information about you and your child will be kept confidential. Your child's medical records will be reviewed in strict confidence by the staff working on this study. Your name will not be used on any of the study documents or on the stored samples or in any reports or publications about this study.

Whom do I contact if I have questions or complaints?

If you have any questions about the study you can contact Dr Le Thanh Hai at the National Hospital for Pediatrics. His phone number is 098 906 3658. If you have any questions about being in a research study you can contact the Clinical Research Office of the Hospital for Tropical Diseases at 083924 1983.

Consent from the parent, guardian or legal representative:

I have read this consent form OR this consent form has been read to me.

[Delete which does not apply]

I have had the opportunity to ask questions about this study and these questions have been answered to my satisfaction. I freely give my permission to allow [write name] to join this study. I understand that I have the right to withdraw him/her from the study. I will be given a copy of this signed consent form to keep for my reference.

I allow / do not allow* samples to be stored for future testing * delete which does not apply.

I allow / do not allow* genetic testing on the stored samples * delete which does not apply.

Name of person giving consent _____

Signature or thumb print _____

Relationship to patient: _____ Date: _____

Person obtaining consent _____

Signature _____

Date: _____

Name witness _____

Signature _____

Date: _____

Assign Patient Study Number: TB _____